Classical swine fever in the pygmy hog

N.N. Barman (1)*, D.P. Bora (1), A.K. Tiwari (2), R.S. Kataria (3), G.S. Desai (2) & P.J. Deka (4)

(1) Department of Microbiology, College of Veterinary Science, Assam Agricultural University, Khanapara Campus, Guwahati-781 022, India
(2) Division of Animal Biotechnology, IVRI, Izatnagar, Uttar Pradesh, India
(3) National Bureau of Animal Genetic Resources, Karnal, Haryana, India
(4) Pygmy Hog Conservation Programme, Basistha, Guwahati, Assam, India

*Corresponding author: nnbarmani@gmail.com

Summary
The pygmy hog is a rare, small and highly endangered mammal belonging to the Suidae family, and it is presently found only in the Assam state of India. While investigating the cause of death of pygmy hogs housed at a conservation centre for captive breeding and research at Basistha, Assam, it was confirmed that they were susceptible to and died as a result of contracting classical swine fever (CSF), caused by CSF virus (CSFV), which is a highly infectious endemic disease of domestic pigs in India. The post-mortem findings and serum CSFV-specific antibody titres, along with the isolation of CSFV from two pygmy hogs, and further confirmation by CSFV genomic E2 and 5’ untranslated region (UTR) gene amplification in PCR (polymerase chain reaction), clearly established the cause of death of the pygmy hogs. Further, on phylogenetic analysis, the pygmy hog CSFV 5’ UTR sequences were grouped in the genotype 1.1 cluster of Indian CSFVs, and hence the strains causing infection were closely related to CSFV isolates circulating in domestic pigs. Therefore, the occurrence of CSF in this endangered species may pose a potent threat to their existence unless properly controlled, and thus it needs urgent attention. To the authors’ knowledge this is the first report on CSF in pygmy hogs.

Keywords

Introduction

Of the many animal viral diseases that are notifiable to the World Organisation for Animal Health (OIE), classical swine fever (CSF) is one of the most important global diseases of Suidae, and it is caused by the CSF virus (CSFV), which belongs to the *Pestivirus* genus within the virus family *Flaviviridae*. The disease occurs in most parts of the world except North America and Australia. The CSFV is a small, enveloped particle containing a single-stranded RNA genome of 12.5 kb. The single open reading frame (ORF) of the CSFV genome encodes 11 or 12 final cleavage products, and is flanked by highly conserved 5’ and 3’ untranslated regions (UTRs). The structural components of CSFV are formed by C, E<sub>pro</sub>, E1 and E2 proteins (34). Although CSF has been eradicated in domestic pigs in some parts of the world, a reservoir is maintained in wild boar populations and the virus can be reintroduced into domestic pigs, resulting in fresh outbreaks and spread of the disease (2, 17). Since about 77% of infectious diseases of domestic animals are common to wildlife (15), a highly contagious disease such as CSF can cross over rapidly from domestic animals to captive or free-ranging wild animals in neighbouring habitats through contact with infected domestic pigs, contaminated feed or water, or through fomites and humans, and these diseases pose a great threat to the existence of the rarest wild fauna.

The pygmy hog (*Sus salvanius*), recently reclassified as *Porcula salvania* (9), is the rarest wild suid in the world and is regarded as the closest relative of the Eurasian pig *Sus scrofa* (11, 19). Today, it is on the brink of extinction, as the only viable wild population of this species exists in the Manas National Park, near to the capital city of Guwahati, in the Assam state of India (18). The International Union for Conservation of Nature (IUCN) has accorded the highest priority rating status to the pygmy hog and included it in the list of critically endangered
species (category 6). Therefore, to conserve this most endangered species, a pygmy hog research and breeding centre has been set up at Basistha, Guwahati, Assam, India. In the captive breeding stock, stringent hygiene measures, including isolation from direct contact with other animals, have been adopted (20). Despite this, the pygmy hogs have been infected with various infectious diseases, including bacterial, fungal and parasitic, as well as viral infections (3, 18); many of the diseases that affect the pygmy hogs have yet to be characterised. An outbreak of salmonella was recorded in the pygmy hog by Rahman et al. (24). Prior to the outbreak of CSF, association of a disease with a causative viral agent was not thoroughly investigated. However, during the years 2003 to 2005, there was some mortality in the animals and, in an attempt to diagnose the cause of death, tissue samples from dead and ailing animals were screened for CSF. The study indicated that the pygmy hogs had sub-acute or acute CSF, and some animals succumbed to the virulent disease. To the authors’ knowledge, this paper is the first to clearly establish that CSF poses an infectious disease threat to the continuing existence of the pygmy hog.

Materials and methods

History and source of materials

Two male and four female wild hogs were captured from the Manas National Park, Assam, India, in March 1996, and were housed in the breeding centre at Basistha, Guwahati, Assam (20), for captive breeding following routine husbandry practices. The animals in the centre are provided with hygienically prepared feed twice daily, and clean drinking water ad libitum. A stringent sanitation and hygiene procedure is adopted. Footbaths are provided at all entry points and the area is totally out of bounds to any stranger. The location of the breeding centre is on the slope of a hilly area; bushy plants and reeds have been grown to create a natural atmosphere. In captivity, the six foundation animals have bred, and the total population of pygmy hogs has risen considerably. Between 2001 and 2005, the hogs of different age groups were infected without showing any visible clinical signs of disease. In some cases, discovery of infected animals was made difficult because they prefer to hide inside bushy plants. A total of 45 infected animals were attended, of which 24 animals were found dead. The remaining animals (mostly adults) were separated, and given treatment with antibiotics and supplements.

In each case where an animal died, a post-mortem examination was performed 2 h to 6 h after death. External body surfaces and internal organs were examined thoroughly, and sizes and numbers of lesions were recorded. A total of 58 clinical samples, from the tonsils, mesenteric lymph nodes (MLNs), kidney, spleen and liver, were collected aseptically for further investigation.

Detection of viral antigen/antibodies

The tissue samples collected at post-mortem between 2003 and 2005 were processed using standard procedures described elsewhere (38) for preliminary detection of CSFV antigen. Subsequently, suspected cases of CSF were investigated regularly for demonstration of CSF viral antigen. Two tests, namely, a polyclonal-antibody-based sandwich enzyme-linked immunosorbent assay (ELISA) and direct fluorescent antibody technique (FAT), were used following the methods of Sarma and Sarma (29) and the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (38) for detection of viral antigen. For demonstration of CSFV antibody in recovered as well as in-contact hogs, paired sera samples were collected. Again, in 2009, a batch of post-weaned hogs was screened for the presence of CSFV antibody.

Sandwich enzyme-linked immunosorbent assay

The test was carried out in 96-well microtitre plates (Maxisorp, Nunc, Denmark). The wells were coated with 50 µl of rabbit anti-CSFV antibodies (1:3000) in 0.05 M carbonate-bicarbonate buffer. The wells were coated overnight and then washed three or four times with wash buffer (0.5 M PBS + 0.05% Tween 20, PBS-T). Wells were preblocked with 50 µl blocking buffer (PBS-T + 5% lactalbumin hydrolysate [LAH] + 10% horse serum) and incubated at 37°C for 1 h. Washing was repeated as described above. Serial dilutions of test samples were made in PBS-T, with an initial dilution of 1:2, and then incubated. After thorough washing, 50 µl of pig anti-CSF antibodies (1:800 in blocking buffer) was added to each well and incubated. After thorough washing, anti-pig horseradish peroxidase conjugate (Dako, Glostrup, Denmark; diluted 1:1000 in blocking buffer), substrate (OPD; Sigma-Aldrich, St Louis, MO, USA) and H₂O₂ were added, and allowed to react. The reaction was stopped with 1 M H₂SO₄. The optical density (OD) was read at a wavelength of 490 nm in an ELISA reader (Bio-Rad, Hercules, CA, USA). An appropriate positive antigen control (laminised CSF vaccine virus) and a negative antigen control (spleen suspension of caesarean-derived day-old piglet and screened as negative for Pestivirus in RT-PCR [reverse transcriptase polymerase chain reaction]) were used in the test. The cut-off OD value for each sample was calculated by subtracting the mean OD value of the negative control from the mean OD value of the test sample. A difference of ≥ 0.1 in the OD value of highest dilution between the negative control and the test sample was considered to be a positive result.
Direct fluorescent antibody technique

Tissue sections (approximately 5 µm to 6 µm thick) were prepared using a cryotome (Thermo Shandon, Runcorn, UK) and the slides were fixed in chilled acetone (−20°C) for 10 min. Cryosections were thawed, briefly washed in washing buffer, and a circle was drawn around sections using a PAP Pen (Dako). A 50 µl volume of pig anti-CSFV fluorescein isothiocyanate (FITC) conjugate (Community Reference Laboratory, Hanover, Germany; 1:10 dilution) was put onto each section, and the sections were incubated at 37°C for 30 min in a humid chamber. After thorough washing, mounting buffer was applied to each slide, and the sections were covered with coverslips. Each test section was compared with a negative section to assess the background stain. The slides were examined under a fluorescence microscope (Karl Zeiss, Germany). Cells exhibiting bright green fluorescence were identified as virus-infected cells.

Indirect enzyme-linked immunosorbent assay

The development and titre of antibodies in serum samples from the pygmy hogs that had recovered from infection, as well as those in contact with the disease, were determined by the indirect ELISA, following the method of Sarma and Sarma (29), with a slight modification. The test was carried out in a 96-well flat-bottomed polystyrene plate (PolySorp, Nunc, Rochester, NY, USA). The wells were coated with purified vaccine antigen (1:500). Two-fold serial dilutions of the test serum were made, with an initial dilution of 1:10 in blocking buffer containing 5% LAH in 0.5 M PBS-T. A 50 µl volume of rabbit anti-swine horseradish peroxidase conjugate (1:1000; Dako) was added into each well, and the plates were incubated. After a thorough washing, 50 µl of freshly prepared substrate (H2O2) and chromogen (orthophenyldiamine; Sigma-Aldrich) mixture was added to the wells and allowed to react for 15 min. The reaction was stopped by adding 50 µl of 1 M H2SO4 to each well. Positive hyperimmune serum and negative serum samples were run as controls in each plate. The highest dilution of the test well showing a corrected OD value (OD of test well minus mean OD of negative control) of ≥0.1 was considered to be the titre of the serum sample.

Isolation of classical swine fever virus

Samples found to be positive for CSFV in ELISA and FAT were processed for isolation of CSFV in cell culture. The virus was isolated in the established PK15 cell line. A tissue culture flask containing a confluent monolayer of PK15 cells in the seventh passage was obtained from the National Centre for Cell Science, Pune, India. The cells were maintained in the laboratory by continuous subculturing following standard procedure (38). The growth medium (Eagle’s minimal essential medium [EMEM] with 10% gamma-irradiated fetal calf serum, free from pestiviruses and their antibodies) was discarded and the cell monolayer was prepared for inoculation with field samples. A 20% tissue suspension was treated with glutamine/antibiotic stock solution: 1 ml per 10 ml tissue suspension. The cell monolayer was inoculated with 0.5 ml of antibiotic-treated sample spread evenly over the entire monolayer of cells by tilting the flask. The inoculated flasks were incubated at 37°C for 30 min for adsorption of viruses to cells. After removal of unadsorbed viruses by washing, maintenance medium (EMEM with 10% fetal calf serum) was added to the flask and incubated at 37°C for 24 h to 36 h. The inoculated flasks were harvested 4 to 5 days later, and stored at −20°C. Each clinical sample was given a minimum of 10 passages in the cell culture. Propagated CSFV in cell culture was confirmed by direct FAT, sandwich ELISA and RT-PCR.

Detection of viral nucleic acid

Reverse transcriptase nested polymerase chain reaction (RT-nPCR) was used for detection of CSFV nucleic acid in various tissue samples, such as tonsil, lymph node, kidney and liver, collected from infected pygmy hogs. Viral RNA was extracted from the tissue suspensions, cDNA was synthesised by reverse transcription, and then specific amplification of CSFV was done by RT-nPCR targeting the E2 and 5’ UTR regions of the CSFV genome.

Extraction of viral nucleic acid

Viral RNA was extracted from tissue samples and standard lapinised virus using RNA-TRI reagent (Ambion, Austin, TX, USA), following the manufacturer’s instructions. Briefly, 20% tissue suspension was prepared manually by homogenising tissue in an appropriate volume of PBS. A 200 µl volume of tissue homogenate suspension was serially diluted and 20% tissue suspension was prepared manually by homogenising tissue in an appropriate volume of PBS. A 200 µl volume of tissue homogenate suspension was transferred to a 1.5 ml microcentrifuge tube, and 1 ml of RNA-TRI reagent was added, followed by vigorous mixing by shaking and pipetting the mixture. Finally, the mixture was incubated at room temperature for 10 min for complete dissociation of the nucleoprotein. Then 200 µl of chloroform was added to the mixture and it was briefly mixed for 15 s before incubation at room temperature for 10 min. The mixture was then centrifuged at 12,000 × g at 4°C for 10 min, and the colourless supernatant was transferred to a fresh microcentrifuge tube. A 500 µl volume of isopropanol was added to the supernatant and it was incubated at room temperature for 10 min. To pellet the viral RNA, the sample was centrifuged at 12,000 × g for 10 min. The RNA pellet was washed once with 1 ml of 75% ethanol and then centrifuged again at 7,500 × g at 4°C for 5 min. The supernatant was removed, and the RNA pellet was air dried at room temperature and resuspended in 20 µl of nuclease-free water. Extracted RNA samples were labelled correctly and stored at −20°C until further use.
Primers

Four different sets of primers (Table I) were used in the study. The primers were designed by Lowings et al. (16) and Greiser-Wilke et al. (10) targeting CSFV-specific E2 and pan-pesti-specific 5′ UTR genes, based on the previously published sequence of CSFV Alfort-187 strain. Out of these four sets of primers, two sets (CSF 1.3 and CSF 1.4 for E2, and CSF 2.3 and CSF 2.4 for the 5′ UTR region) were used for nested PCR.

Reverse transcriptase polymerase chain reaction

Complementary DNAs (cDNAs) of viral RNA were synthesised using 6 µl of viral RNA and 1 µl of random primer (50 ng/µl) in a total reaction volume of 13 µl. The contents were incubated in a thermal cycler (PerkinElmer, Waltham, MA, USA) at 70°C for 5 min and 25°C for 10 min. The reverse transcription was carried out by adding annealed RNAs to the reverse transcription mixture consisting of 4 µl of 5x RT buffer, 1 µl RNAase inhibitor (40 U/µl), 1 µl of 10 mM deoxynucleotide triphosphate (dNTP) mixture, and 1 µl of Moloney murine leukaemia virus reverse transcriptase (200 U/µl). The PCR protocol consisted of primer annealing at 25°C (10 min), extension at 42°C (1 h), and inactivation of the enzyme at 70°C (10 min). The cDNAs were stored at −20°C until required for further use.

PCR was carried out for amplification of both E2 and 5′ UTR regions of CSFV following the method described previously (10, 16). The PCR mixture in a total volume of 50 µl, containing 5.0 µl of 10× buffer, 3.0 µl of 25 mM MgCl2, 1.0 µl of primers, 1.0 µl of 10 mM dNTPs, 5.0 µl of cDNA, 0.5 µl of Taq DNA polymerase (Qiagen) and 33.5 µl of nuclease-free water, was subjected to amplification in a thermal cycler at 95°C for 2 min (one cycle), 95°C for 30 s, 56°C for 45 s, 72°C for 1 min (34 cycles), and 72°C for 1 min. For amplification of the 5′ UTR, the annealing temperature was kept at 50°C.

Nested polymerase chain reaction of the E2 and 5′ untranslated regions

For nested PCR of the E2 region gene and the 5′ UTR gene, the procedure was essentially the same as described above, except that the template cDNA was replaced by 5 µl of primary PCR amplicons and the annealing temperature was kept at 58°C for the E2 gene and 56°C for the 5′ UTR gene. The primers used were internal primers CSF 1.3 and CSF 1.4 for the E2 region gene, and CSF 2.3 and CSF 2.4 for the 5′ UTR gene. After amplification, a 5 µl aliquot was electrophoresed through a 1.7% agarose gel and stained with ethidium bromide for visualisation of the expected amplicon. Lapinised CSFV vaccine virus was used as a positive control, and spleen tissue of a piglet delivered by caesarean section was used as a negative control.

Cloning and sequencing of polymerase chain reaction products, and analysis of sequence data

The amplicons of the 5′ UTR gene generated by nested PCR were subjected to purification using QIAquick PCR purification kit (Qiagen, New Delhi, India) and cloned into a pGEM-T Easy vector (M/s Promega Corp., Madison, WI, USA) for sequencing in an automated sequencer (ABI Prism 3100; ABI, IL, USA). Sequences were analysed by comparison with sequences of different pestiviruses available in the GenBank database using the online BLAST server (1). Multiple sequence alignment was carried out by using the ClustalW algorithm (35) and manual editing. Phylogenetic analysis was conducted using MEGA version 4.0.2 (32). For comparison, sequences of various reported CSFV field isolates, and vaccine strains from India and

<table>
<thead>
<tr>
<th>Primer designation/orientation</th>
<th>Genomic region</th>
<th>Primer sequence</th>
<th>Nucleotide position</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF1.1 (forward)</td>
<td>E2</td>
<td>5′-AGRCAGACTGGTGGCCNTAYGA-3′</td>
<td>2228–2250</td>
<td>Lowings et al., 1996 (16)</td>
</tr>
<tr>
<td>CSF1.2 (reverse)</td>
<td>E2</td>
<td>5′-TTYACCATCCTGTTCTA-3′</td>
<td>2898–2880</td>
<td>Lowings et al., 1996 (16)</td>
</tr>
<tr>
<td>CSF1.3 (forward) Internal (CSFV specific)</td>
<td>E2</td>
<td>5′-TCRWCAACCAAYGAGATAGGG-3′</td>
<td>2477–2497</td>
<td>Lowings et al., 1996 (16)</td>
</tr>
<tr>
<td>CSF1.4 (reverse) Internal (CSFV specific)</td>
<td>E2</td>
<td>5′-CACAGYCCRAAYCCRAAGTAC-3′</td>
<td>2748–2726</td>
<td>Lowings et al., 1996 (16)</td>
</tr>
<tr>
<td>CSF2.1 (forward)</td>
<td>5′ UTR</td>
<td>5′-CTAGGACATGGCCWYATAGG-3′</td>
<td>94–113</td>
<td>Greiser-Wilke et al., 1998 (10)</td>
</tr>
<tr>
<td>CSF2.2 (reverse)</td>
<td>5′ UTR</td>
<td>5′-CAGCTTCAARTGATTAGT-3′</td>
<td>514–496</td>
<td>Greiser-Wilke et al., 1998 (10)</td>
</tr>
<tr>
<td>CSF2.3 (forward) Internal (pan-pesti-specific)</td>
<td>5′ UTR</td>
<td>5′-AGCTCAGCTGGTGTGCTCA-3′</td>
<td>146–163</td>
<td>Greiser-Wilke et al., 1998 (10)</td>
</tr>
<tr>
<td>CSF2.4 (reverse) Internal (pan-pesti-specific)</td>
<td>5′ UTR</td>
<td>5′-TGTTTGCTTGTGGTTGATA-3′</td>
<td>417–399</td>
<td>Greiser-Wilke et al., 1998 (10)</td>
</tr>
</tbody>
</table>

CSF: classical swine fever
other parts of the world, were downloaded from the GenBank database.

Results

Pathology of classical swine fever virus infection in the pygmy hog

During the period of 2003 to 2005, out of a total of 45 cases attended, 24 pygmy hogs with suspected CSF were subjected to post-mortem examination, the remaining 21 animals were clinically affected, but recovered, and were investigated for demonstration of CSFV-specific antibody. The majority of affected animals who died were found dead in the morning. Only a limited number of hogs (8 of 45) showed prominent clinical signs of infection, and this was for a period of 2 to 3 days. The signs were characterised by a high rise in temperature (105°C), anorexia, conjunctivitis, hyperaemia (five animals), occasional vomiting (three animals) and constipation. Some affected animals exhibited respiratory (four animals) and nervous distress (two animals). Some of the piglets developed hindlimb paralysis (two animals). Affected animals did not appear to huddle together, but they burrowed under leaves. Others died without showing any clinical symptoms.

Post-mortem examination of dead pygmy hogs revealed various gross changes in the lymphoid organs, kidney, heart, epiglottis, gallbladder, urinary bladder, brain and intestine. Animals below 2 months of age showed haemorrhage and congestion in the heart, kidney and spleen. Tonsils and MLNs were swollen and congested. Prominent pathological changes in grower piglets were congestion and haemorrhage in tonsils, MLNs, spleen and brain. The kidney showed pinpoint haemorrhages in the subcapsular surface (Fig. 1). Haemorrhagic changes were also observed in the myocardium. The intestine was highly congested and showed focal necrosis in the colon.

Detection of classical swine fever virus antigen and antibody

A total of 58 tissue samples were processed for demonstration of CSFV antigen by direct FAT and by sandwich ELISA, the results are presented in Table II. Direct FAT detected CSFV antigen in 12 out of 58 tissue samples (20.7%), while sandwich ELISA showed that 15 of 58 were positive (25.9%). The tissue samples with the highest proportion of positive results were the lymph nodes (8/9, 88%) and the tonsils (4/8, 50%), followed by the spleen samples (9/21, 42%) and kidney samples (6/14, 42%). Samples received between 2005 and 2009 were screened regularly for demonstration of CSFV antigen and antibody. An indirect ELISA was used to screen animals that had been in contact with the disease, as well as those that had recovered, for the presence of CSFV-specific antibody. A total of 21 animals belonging to different age groups were screened and 33.3% (7/21) of these were found to possess CSFV antibody. Animals at 1 month of age were highly seropositive (5/6, 83.3%), with a maximum seroconversion titre of 1:128. Four years after the last recorded outbreak of CSFV (2005), post-weaned young pygmy hogs (13 animals) showed no CSFV-specific antibody in their sera samples. Tissue samples (20 samples) examined, irrespective of the presence of typical lesions, did not indicate the presence of CSFV antigen.

Polymerase chain reaction

Nested PCR amplification of both E2 and 5′ UTR regions, using CSFV-specific primers (Table I), showed the expected product of 271 bp (Figs 2a and 2b) in both of the genes. Out of 58 tissue samples collected, 18 (31.03%) samples showed the presence of CSFV antigen. The remaining samples were found to be negative for CSFV.

<table>
<thead>
<tr>
<th>Source of sample</th>
<th>No. of samples tested</th>
<th>No. of samples positive (%)</th>
<th>FAT</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tonsils</td>
<td>9</td>
<td>2 (22.2)</td>
<td>2</td>
<td>2 (22.2)</td>
</tr>
<tr>
<td>Mesenteric lymph nodes</td>
<td>9</td>
<td>4 (44.4)</td>
<td>4</td>
<td>4 (44.4)</td>
</tr>
<tr>
<td>Spleen</td>
<td>21</td>
<td>3 (14.3)</td>
<td>6</td>
<td>28.6</td>
</tr>
<tr>
<td>Kidney</td>
<td>14</td>
<td>3 (21.4)</td>
<td>3</td>
<td>(21.4)</td>
</tr>
<tr>
<td>Liver</td>
<td>6</td>
<td>0 (00.0)</td>
<td>0</td>
<td>0 (00.0)</td>
</tr>
<tr>
<td>Total</td>
<td>58</td>
<td>12 (20.7)</td>
<td>15</td>
<td>(25.9)</td>
</tr>
</tbody>
</table>

ELISA: enzyme-linked immunosorbent assay
FAT: fluorescent antibody test

Fig. 1
Subcapsular region of classical swine fever virus infected kidney of the pygmy hog shows pinpoint haemorrhages
Isolation of classical swine fever virus

A total of six clinical and post-mortem samples found to be positive in FAT, S-ELISA and PCR were processed for isolation of CSFV. Samples consisted of tonsil ($n = 2$), spleen ($n = 2$) and kidney ($n = 2$). The CSFV propagated in the PK15 cell monolayer and passaged up to sixth passage level did not exhibit any detectable cytopathic effect (CPE).

Phylogenetic analysis of the 5′ untranslated region gene

The nested PCR amplicons of the 5′ UTR gene of CSFV from pygmy hogs (PH D 2004 and PH 13/2005) were cloned into the pGEMT vector and sequenced commercially. Sequence analyses, based on 151 nucleotides of the 5′ UTR gene of the pygmy hog isolates, were found to be identical to other 5′ UTR genes of CSFV isolated from different parts of the world, except for a difference in one nucleotide (G in PH 13 and A in PH D) at nucleotide position 52 of the 150 bp 5′ UTR region. The 5′ UTR sequences generated in this study (Table I) have been submitted to GenBank (GenBank accession numbers EF471209 and EF471210).

Discussion

The ‘spillover’ (transmission of infectious agents from reservoir domestic populations to sympatric wildlife) of epizootic outbreaks represents a serious threat both to wildlife and, via ‘reverse spillover’, to sympatric populations of susceptible domesticated animals (6). Also, the potential transfer of pathogens into previously
Fig. 3
Phylogenetic tree showing relationship of pygmy hog classical swine fever virus (CSFV) isolate with other CSFV based on the 5' untranslated region gene

The pygmy hog isolates are highlighted with buttons (PH 13/2005 and PH D/2004)
unexposed wild populations in often sensitive, protected areas, such as those designated for captive breeding programmes, represents a serious challenge to conservation efforts (37). According to the IUCN Red List of Threatened and Endangered animals, of the 733 animal species that are known to have become extinct over the past 500 years because of multiple causal factors, 31 cases have been attributed, at least in part, to infectious diseases. The IUCN Red List also reports that of the total 2,852 critically endangered species (1,490 plants and 1,362 animals), 233 (8% of the total) have infectious diseases listed as a contributing threat.

The pygmy hog is a rare Suidae species with high genetic variability, and it is listed in the IUCN Red List in the ‘Critically Endangered C2a’ category (version 3.1) (www.iucnredlist.org/apps/redlist/details/21172/0). The animal is regionally extinct in Bangladesh and Nepal, and its presence is uncertain in Bhutan. In India, the pygmy hog is now confined to a very few locations in and around Manas National Park in north-western Assam (18). The major threats listed for the ‘critically endangered species’ status of the pygmy hog in the IUCN Red List are similar to the threats for any wild fauna of the same status, and they include loss and degradation of habitat because of human settlements and activities. However, as a result of habitat relocation of the pygmy hog in conservation efforts, a little known, and what was thought to be less significant, new threat of infectious diseases to the pygmy hog has been encountered. In 2001, Rahman et al. (24) reported the death of seven (six adult and one young) pygmy hogs at the Research and Breeding Centre, Basistha, and this was found to be due to Salmonella enteritidis infection. However, the source of this infection in the pygmy hog was not attributed to feed or water.

As is widely known, CSF is a highly contagious transboundary animal disease that affects domestic pig and wild boar, and it can rapidly spread across a wide geographical area through the movement of animals and animal products. Transmission through indirect contact, such as feeding green feed/fodder harvested in areas of infected herds, or through contact with infected personnel and vehicles, is an important source of infection (13, 14).

The clinical course of the disease in wild boar is similar to that in domestic pigs, where affected animals show an acute, subacute or chronic form of disease (7). In addition, infected wild boar tend to exhibit markedly altered behaviour, such as loss of natural shyness and roaming during daylight hours (12). In the present study, except for 8 of the 45 pygmy hogs attended, the majority of affected animals did not reveal typical clinical signs of CSF. This might be because they were infected with a virus strain that showed different virulence characteristics (16). Considering the different epidemiological factors, along with persistent virus infection, the clinical signs of CSF can be extremely variable and therefore hamper correct and timely recognition in the field (2). Clinical signs, as well as gross changes, recorded in the present investigation were in accordance with those reported by others (17). It has been stated that the mortality rates in wild boar vary with the clinical course of the disease (26).

Distribution of the viral antigen in the various lymphoid tissues of the pygmy hog reaffirms that CSFV shows a tropism towards lymphoid organs. Van Oirschot (36) stated that almost all lymphoid organs including tonsils, MLNs and spleen were affected in the first viraemic phase, and propagated to highest concentration. On the other hand, the virus probably does not invade the parenchymatous organ until late in the viraemic phase.

Pygmy hogs and domestic pigs appear to have a similar susceptibility pattern to CSFV infection. In both species, the clinical course of the disease, the mortality pattern, and the development of CSFV-antibodies in ‘in-contact’ animals is much the same. The presence of CSFV-specific antibody in animals who recovered from the disease provides supportive evidence regarding the exposure of the breeding stock to the active virus infection. Interestingly, young pygmy hogs and adults showed a high antibody titre. The presence of high levels of CSFV-specific antibody strongly suggested that mothers infected with CSFV transferred the antibody passively to the young animals. Again, the absence of CSFV-specific antibody in post-weaned hogs four years after the last recorded outbreak confirms that the strict hygiene measures adopted subsequently prevented re-infection of the breeding stock with CSFV.

Various workers use the PK15 cell line for isolation of CSFV (23, 27, 33). Moreover, virus isolation is considered to be the gold standard for confirmation of a CSFV outbreak (2). In the present study, samples processed for isolation were found positive in the fifth passage. In the propagated samples, one sample showed highly positive results in FAT, S-ELISA and PCR. From these results it was observed that for adaptation of CSFV to cell culture at least four to five blind passages are required. Singh (31) also reported a similar observation, and suggested that for adaptation of CSFV to cell culture at least two to three blind passages are required.

For CSF diagnosis, antigen detection methods such as FAT and ELISA have lower sensitivity and specificity, but they have great advantages owing to their simplicity and rapid turnaround times (2) compared with other antibody-based techniques, i.e. neutralisation tests. In the present investigation, the two tests showed similar observations with respect to their performance in the detection of CSFV antigen in tissue samples.
Along with the antibody-based detection, RT-nPCR amplification of E2 and 5′ UTR regions of CSFV confirmed the involvement of the CSFV in the pygmy hog. Although polyclonal-antibody-based diagnostics (S-ELISA, FAT) are able to detect members of the Pestivirus group as a whole (5), amplification of the nucleotides encoding E2 epitope using current primer sets is specific for CSFV (16). This region is responsible for synthesising the most immunogenic glycoprotein of the virus (4, 25). The lapinised strain of CSFV used in this study as a known positive control also gave a comparable band in agarose gel electrophoresis. Nested RT-PCR was found to be more sensitive than FAT or S-ELISA in detecting CSFV. Paton et al. (22) have also reported the order of test sensitivity as RT-nPCR > RT-PCR > virus isolation > ELISA in detection of CSFV.

The nucleotide sequences of fragments of the 5′ non-coding region (5′ UTR), the E2 envelope glycoprotein and the non-structural protein NS5B are useful for classification of CSFV into groups and subgroups (16, 22). A phylogenetic tree constructed by the neighbour-joining method for 5′ UTR sequences of 50 Indian CSFV isolates along with the vaccine strain (VAC-IVRI) has been reported in recently published papers (8, 21) and the pygmy hog CSFV isolates of the present study all grouped into genotypic clusters 1.1 (Fig. 3). Also, this phylogenetic clustering indicated clearly that the pygmy hog isolates must have been derived from and closely related to CSFV isolates already circulating in domestic pigs.

Since the first reported outbreak of the disease in 1962 (28), CSF has been an animal disease of major economic significance, and it is endemic in domestic (indigenous, crossbred and exotic breeds) pig populations in India. Between the years 1996 and 2008, almost 1,308 outbreaks of CSF were reported from different parts of India (OIE World Animal Health Information Database: www.oie.int). However, there have been no reports of CSF outbreaks in Indian wild boar and hence their role in the epidemiology of CSF is not clearly known. Seven north-eastern states, including the state of Assam, are home to more than almost a quarter of India’s pigs. The majority of the pigs in Assam (over 1.5 million) are indigenous and crossbreds reared in backyards using traditional husbandry practices. Local forest products contribute to the state’s economy and play an important role as a source of feed for livestock. Hence, considering the presence of a high density of CSFV-susceptible domestic pigs and the endemic epidemiology of CSF in these animals in Assam (24, 30) and other neighbouring states, the authors predict the possibility of indirect transmission of CSFV to pygmy hogs by infected personnel or vehicles entering the captive breeding habitat, as the chance of direct transmission is extremely rare in the protected housing and breeding programme. However, the role of wild birds in indirect spread of CSF to the protected habitat cannot be ruled out. Extensive molecular epidemiological studies of CSFV isolates from different regions of India could give a clear picture of the evolutionary trend, and the distribution of various groups and subgroups of CSFV in this country.

**Conclusion**

From this study it can be concluded that: (i) pygmy hogs are highly susceptible to CSF infection; (ii) CSF causes death of affected pygmy hogs; (iii) CSF might have been transmitted to pygmy hogs from the infection in local/domestic pigs; (iv) there is a possibility of CSF infection resulting in the death of more pygmy hogs in future, as more and more animals become exposed in the protected habitat; (v) there is a possibility that the chronically infected pygmy hogs could become a source of infection in domestic pigs and other feral pig populations once they are released into the wild. Therefore, there is an urgent need for an extensive epidemiological study of CSF so as to formulate a strategy to protect this unique creature.

**Acknowledgements**

The authors would like to thank the Department of Biotechnology, Government of India, for providing financial help to carry out the research under the project entitled ‘Molecular and immunological characterisation and phylogenetic analysis of Indian isolates of classical swine fever virus’. They would also like to thank the Pygmy Hog Conservation Programme (Research and Breeding Centre for Pygmy Hog Conservation, Basistha, Assam, India), for supplying the samples and additional support.
La peste porcina clásica en el jabalí enano


Resumen
El jabalí enano es un pequeño y raro mamífero de la familia de los suidos que actualmente está amenazado y solo se encuentra en el estado de Assam (India). Al investigar las causas de la muerte de jabalíes enanos de un centro de protección situado en Basistha (Assam) y dedicado a la investigación y la cría en cautividad, se comprobó que esos animales eran susceptibles al virus de la peste porcina clásica (VPPC) y morían a causa de esa enfermedad.
extremadamente infecciosa, endémica en la población de cerdos domésticos de la India. Los análisis post-mortem y los títulos séricos de anticuerpos específicos anti-VPPC, así como el aislamiento del virus en dos jabalíes enanos y una nueva confirmación por amplificación génica (mediante PCR) del gen codificador de la proteína E2 y de la región terminal 5’ no traducida del genoma vírico, sirvieron para determinar con certeza la causa de la muerte de los jabalíes enanos. Además, por análisis filogenético, se agruparon las secuencias 5’ no traducidas del VPPC del jabalí enano en el grupo genotípico 1.1 de los virus indios de la PPC, de donde se sigue que las cepas causantes de la infección estaban muy emparentadas con los VPPC circulantes aislados en cerdos domésticos. Por consiguiente, y a menos que se le ponga coto adecuadamente, la aparición de PPC en esta especie amenazada puede poner en grave peligro su existencia, por lo que urge prestar atención al problema. Hasta donde saben los autores, es la primera vez que se describe la presencia de PPC en jabalíes enanos.

Palabras clave

References


