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Clinico- pathology and diagnosis of CSF in Zovawk pigs: An indigenus pig of Mizoram, India

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Classical swine fever (CSF) is a highly contagious and economically important viral disease of both domestic pigs and wild boars. The causative agent, classical swine fever virus (CSFV), is a member of the genus pestivirus in the family *Flaviviridae*. The CSFV genome is a positive polarity RNA of about 12.3kb in length which contains un-translated regions (UTR) at 5' and 3' ends and encodes a single polyprotein that is both co- and post- translationally processed to yield 4 structural (C, EO, E1 and E2) and 7–8 nonstructural (Npro, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) viral proteins (Meyers and Theil 1996, Patton and Greiser-Wilke 2003).

CSF is highly prevalent among swine in Asian countries, central and south-America and in some wild boar populations in EU (Van Gennip *et al*. 2002, Laddomada *et al*. 1994).Although vaccination programs were implemented in pig farms of India for many years, cases of mild symptoms or even severe outbreak were frequently reported, and it is still an epidemic disease in India. This article reports clinical manifestation, pathology and diagnosis of the disease in Zovawk, an indigenous breed of pigs of Mizoram, India.

Clinico-pathological examination: Affected pigs were observed for clinical signs and appearance of gross lesions. A standard format was prepared for recording and scoring of clinical signs and gross lesions exhibited by individual animals as given in Table 1.

Detail post-mortem examination of carcasses was carried out in dead pigs and gross pathological changes observed in different organs were systematically recorded. Representative tissue samples were collected from tonsils, lymph-node, kidney, spleen, brain, lungs, heart, colon and distal part of ileum. Tissue samples were preserved in 10% formalin solution for histopathological examination. After proper fixation, the tissues were processed, embedded in paraffin and 4–5 µ thick sections were made and stained with routine Haematoxylin and Eosin for histopathological examination (Luna 1968). Representative tissue samples were also preserved at –80°C for molecular detection of CSFV.

Table 1. Score card to record clinical signs (0–3 scale:-0– normal; 1-slight alteration; 2-distinct alteration; 3-severe alteration)

Clinical signs	0	1	2	3
Dullness/depression	Normal	Mild	Moderate	Severe
Respiratory problem	Normal	Mild	Moderate	Laboured breathing
Gait	Normal	Mild	Mild incoordination	Staggering gait
Skin (petechie / haemorrhages)	Normal	Mild	Few petechie	Multiple
Eyes (conjunctivitis)	Normal	Absent	Moderate	Severe
Appetite/anorexia	Normal	Mild	Moderate reduction	Completely off-feed
Diarrhea/constipation	Normal	Mild diarrhoea	Watery faeces	Fibrin Mix/hard pellet
Fever	Normal	Mild (38–39.5°C)	Moderate (39.5–40.5°C)	High (Ã40.5°C)
Fibrin on faeces	Normal	Absent	Moderate	Severe
Ear cyanosis	Normal	Absent	Moderate	Severe
Runting /wasting	Normal	Mild	Moderate	Severe
Nasal discharge	Normal	Mild	Moderate	Severe

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RNA extraction and RT-PCR: Total RNA was extracted using TRIzol reagent according to the manufacturer's protocol. Reverse transcription reactions were performed using random primer with the cDNA synthesis kit as per the

Table 2. List of primers

Primer Identity	Sequence	Product size (expected)	Annealing temp. (standardized)
NTRF	5‘-CTA GCC ATG CCC WYA GTA GG 3‘	421 bp	50 ºC
NTRR	5‘-CAG CTT CAR YGT TGA TTG T-3‘		
E2F	5‘-AGR CCA GAC TGG TGG CCN TAY GA-3‘	671 bp	55 ºC
E2R	5‘-TTY ACC ACT TCT GTT CTC A-3‘		
E2IF	5´TCR WCA ACC AAY GAG ATA GGG 3´	271bp	55 ºC
E2IR	5´CAC AGY CCR AAY CCR AAG TCA TC 3´		
NS5BF	5‘-GAC ACT AGY GCA GGC AAY AG-3‘	449 bp	56 ºC
NS5BR	5‘-AGT GGG TTC CAG GAR TAC AT-3		
ERNSF	5‘-CATGCCATGGCCCTGTTGGCTTGGGCGGTGATA-3‘	735bp	65 ºC
ERNSR	5‘-GGAATTCTCAGGCATAGGCACCAAACCAGG-3‘		

protocol given by the manufacturer. Genomic region that encode the 5´ NCR (Greiser-Wilke *et al*.1998), NS5B (Bjorklund *et al*.1999), E^{rns} (Meyers *et al*. 1999) and E2 (Lowings *et al*.1996) glycoprotein of CSFV were amplified by PCR, using the following primer sets (Table 2).

For 5´ NTR, E2(671 bp),NS5B and E^{rns} gene fragments, PCR was performed with 5pg -.5µg cDNA in a 25µl volume of 10X Taq buffer 2.5µl, 2mM dNTP .25µl, 20pmol of each primer, 1.25units Taq DNA polymerase. Cycling parameters were 95°C×2min, 95°C×1 min, optimal annealing temperature, 72°C×1 min and 72°C×5 min. For E2 inner fragment (271bp) a nested PCR was performed with the PCR product of the outer E2 fragment (671 bp) by using same thermal profile. The products were checked in 1.5% agarose gel electrophoresis.

Outbreak of CSF in a pig farm rearing Zovawk pigs, with a total population of 83 was studied. Zovawk (*Zo* stands for hills or tribal land/people and *Vawk* stands for pig) is an indigenous breed of pigs available in Mizoram and Myanmar, characterized by its black body colour with sometimes white patches on belly and star mark on forehead, pointed snout, bulging or hanging belly and erected ears. Zovawk are mud loving breed of pig, alert, energetic, wildly and good scavenger with their pointed snout. The breed is very popular for its lean meat and almost every household in Mizoram, rear this pig in their backyard.

A total of 42 (50.60%) ((4 adult: 1 male + 3 females), 26 post weaned pigs in the age group of 2–4 months (8 male + 18 female) and 12 pre-weaned pigs in the age group of 1– 2 months (6 male + 6 females)) pigs died in 3 months. Affected pigs were clinically studied and scoring was made as per the standard performa. Out of 42 clinical CSF cases, 22 (55%) cases showed clinical score of 3 (acute case/severe). The affected pigs showed high rise of temperature (103–106°F). Animals were depressed, inactive and completely off fed. The affected piglets huddled together in the corner of the house. Conjunctivitis and thick ocular discharge were noticed in all the affected animals. Four of the affected animals showed complete adhesion of the eye lids due to sticky ocular discharges. Animal showed laboured breathing with watery nasal discharge and exhibited swaying movement of the hind quarters. Affected pigs died within 3–5 days from the onset of clinical signs.Three pigs (2 pregnant sows and one 5-month-old male pig) died suddenly without showing any clinical signs. In all the acute cases studied, the pigs showed multiple petechial haemorrhages on the skin or purple discolouration of skin in ventral abdominal region and in the medial side of the legs.

A total of 16 (30.09%) cases studied showed clinical score of 2 (chronic/moderate) where the affected animal showed dullness, pyrexia and intermittent diarrhoea lasting up to 15–20 days. At the time of death affected pigs were very much debilitated, hide bound and anaemic. Four (9.52%) cases showed clinical score of 1 (atypical/mild form). Affected pigs suffered from intermittent diarrhoea, mild fever, in appetence, retarded growth and survived more than 1 month. Similar clinical signs were also recorded by earlier workers (Dahle and Liess 1992, Sarma and Sarma 1998, Rahman *et al*. 2001, Barman *et al.* 2003, Dutta *et al.* 2003,Moennig *et al.* 2003). Variation in the clinical manifestation of the disease may be due to different strains of CSFV involved in causing the disease. Van Oirschot (1999) proposed that depending upon the virulence and pathogenecity of the viral strain, there may be variability in the clinical picture of the disease with highly virulent strain killing nearly all pigs. The moderate virulent strains cause sub- acute illness in post natally infected piglets and abnormalities in porcine foetuses. The avirulent strains are virtually apathogenic for both pigs and foetuses. Besides, host factors like age, immune status and breed of the affected pigs may also influence the clinical form of the disease (Deppner *et al*. 1997).

Postmortem examination of acute cases showed multiple petecheation or purple discolouration of skin in abdominal region, ears and in medial side of legs. Opening of the carcasses revealed subcutaneous echymotic haemorrhages. All the superficial lymph nodes including tonsil were swollen, oedematous, hemorrhagic and dark tan coloured. Typical infarction in the spleen was observed in 8 cases. The spleen was enlarged and congested with small raised haemorrhagic areas. Mesenteric blood vessels were severely congested in

Figs. 1–2. **1.** Oedematous lungs with petecheal haemorrhages and suffusion at many places, Zovawk pig died of acute CSF. **2.** Button ulcers in colon of Zovawk pig died of chronic CSF.

all acute cases. Petechial haemorrhages on the epicardial surface of the heart were also noticed. Liver was dark, enlarged and congested in all cases. Lungs showed severe haemorrhagic interstitial pneumonia. Affected lungs were oedematous with petechial hemorrhages to suffusion (Fig. 1). Pin point or petechial haemorrhages in the sub capsular region of the kidneys resembling 'Turkey egg kidney' were also observed. Cross section of kidney revealed severe congestion in the cortico medullary junction. Similar changes but of milder intensity were observed in chronic cases. Petecheation on epicardial surface of the heart and suffusion in lungs were not seen in chronic cases. Bronchopneumonia with consolidation of lungs and typical button ulcers were observed in large intestine of 5 chronically affected animals (Fig. 2).

The gross lesions of acute CSF results primarily due to severe vascular alterations in various organs leading to haemorrhagic diathesis which produces haemorrhages in kidney, lymph nodes, skin, mucous and serous membrane, as well as spleen infarctions, all of which have been described in numerous research papers (Weide *et al*. 1962, Dahle and Liess 1992,Moenning and Plagemann 1992). In addition, catarrhal, fibrinous and haemorrhagic inflammatory reaction can be observed in respiratory, digestive, nervous and urogenital system (Moenning *et al*. 2003).

Histopathological examination showed haemorrhagic interstitial pneumonia in lungs. The alveolar wall was very much thickened due to edema, severe congestion of blood vessels, haemorrhages and mononuclear infiltration. The lumen of bronchi and bronchules were filled with sero-fibrinous exudates, necrotic debris, desquamated epithelial cells with mononuclear infiltrations. The interlobular septae was thickened due to accumulation of sero fibrinous exudates. Severe congestion and haemorrhages was observed throughout the spleen parenchyma. The periarterial lymphatic sheath (PALS) showed depletion of lymphocytes. Kidney showed haemorrhages in the cortico medullary junction with tubular degeneration. The cortex of the lymph node showed haemorrhages, congestion and lymphoid depletion. Most severe changes were observed in the mesenteric lymph node. Similar changes but of milder intensity were observed in chronic cases. In addition micro abscesses were observed at many places of lung parenchyma with bacterial clumps. Similar observations were described by Saini *et al*. (2000), Rahman *et al*. (2001). Although the microscopic lesions of CSF are well defined, it is necessary to characterize the derangement caused by each field isolate to determine factors such as tissue tropism and distribution in order to be able to elaborate practical criteria for its rapid diagnosis.

The diagnosis of the CSF is generally confirmed by type-specific monoclonal antibodies, particularly those directed against E2 and by PCR targeting relatively conserved region of CSFV genes. The 5´NTR nucleotide sequence is highly conserved among all members within the genus Pestivirus, thus being useful for the characterization of species or genotype. The envelope of pestiviruses contains three glycoprotein E^{rns}, E1 and E2 (Thiel *et al.* 1996). E2 the major glycoprotein is highly immunogenic and induces high virus neutralizing titers of serum antibodies (Hulst *et al.* 1993, Van Rijin *et al.* 1996, Van Zilj *et al.* 1991 and Bouma *et al.* 1999). The CSFV non- structural protein 5B (NS5B) encodes an RNA- dependent RNA polymerase (RdRp) with 70– 75% identical amino acid sequences but still appears to have conserved tertiary structure rather than primary sequence (Zhang *et al*. 2005) and is a valid candidate to CSFV and non- CSFV pestivirus differentiation. In the present study reverse transcriptase – polymerase chain reaction (RT-PCR) on tissue samples from tonsil, lymph nodes and spleen targeting the 5´NCR (421bp), E2 (271bp), NS5B (449bp) and E^{rns}(735bp) gene fragments of CSFV in all the 42 cases revealed positive results for CSFV and confirmed the outbreak of CSF in Zovawk pigs (Fig. 3).

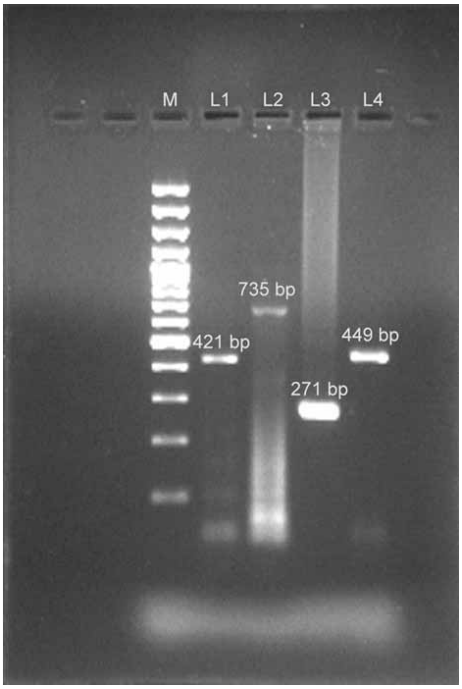

Fig. 3. 1.5% Agarose gel electrophoresis showing amplified product of L1-5'NCR (421 bp), L2-Erns (735 bp), L3- E2 (271 bp) &L4- NS5B (449 bp) gene fragments.

Pig husbandry plays an important role in the socio-economic development in North Eastern states of India, including the state of Mizoram. Department of Veterinary and Animal Husbandry, Government of Mizoram has identified the CSF as the disease of special economic importance for Mizoram (Annual Report, Department of Veterinary and Animal Husbandry, Government of Mizoram 2002–2003). Some of the important factors that have helped perpetuate the disease are lack of adequate doses of vaccine and its availability, timely diagnosis, unrestricted movement of pigs from one region to another within the country and across the porous international border surrounding the North Eastern states and poor public awareness. Thus a detail epidemiological investigation with proper preventive and control measures against the disease is an urgent need to contain the disease.

SUMMARY

Zovawk is an indigenous breed of pigs available in Mizoram and Myanmar, characterized by its black body colour with white patches on belly, white star mark on forehead, pointed snout, bulging or hanging belly and erected ears. The present study reports an outbreak of classical swine fever in a Zovawk pig population, Aizawl, Mizoram. Three different clinical menifestation: acute, chronic and atypical or mild form of the disease was observed in the affected pigs. Postmortem and histopathological examination of the dead pigs showed characteristic gross and microscopical lesions of CSF. The outbreak was further confirmed by RT-PCR on representative tissue samples targeting the genomic region of CSFV that encode the 5´ NCR, NS5B, E^{rns} and E2 gene.

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