RECENT TRENDS IN THE DIAGNOSIS OF CLASSICAL SWINE FEVER

Chakraborty S[1], Choudhury S[2]
1. M.V.Sc (Scholar), Veterinary Microbiology, India
2. Department of Veterianary Pharmacology, Mathura, India
Correspondence: Dr. Sandip Chakraborty. M.V.Sc (Scholar), Veterinary Microbiology, India.
Email: sandipc_85@yahoo.com

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ABSTRACT

Classical swine fever is a disease of pig having significant economic impact. The disease is enzootic in several countries including India. Classical Swine Fever Virus (CSFV) is a difficult virus to work with and major progress only became possible with the development and availability of sophisticated virological methods. The present review deals with the several diagnostic methods to detect the presence of whole virus, viral antigen or antibodies against the virus which ultimately strengthens the prevention and control policies.

Key words: Classical swine fever, diagnosis, recent, trends

INTRODUCTION

Among the various livestock species, pigs are the most potential source of meat production and more efficient feed converters after the broiler chicken. The contribution of pork products in terms of value, works out to be 0.80 per cent of total livestock products and 4.32 per cent of the meat and meat products [1]. As there is ever increasing trend of pork production and consumption in the country, it has become necessary to control some of the economically important diseases in the swine population like Classical swine fever in addition to FMD and Transmissible Gastro-enteritis. Classical Swine Fever (CSF), also known as hog cholera is one of the most dreaded and devastating viral diseases of swine causing serious economic losses directly due to mortality, retardation of growth, reproductive problems and indirectly by bringing restrictions on exports of pork and pork products.

CAUSATIVE AGENT

The disease is caused by Classical Swine Fever Virus (CSFV), a member of the genus *Pestivirus*, family *Flaviviridae*, and is closely related antigenically and structurally to Bovine Viral Diarrhea virus (BVDV) and Border Disease Virus (BDV). It is an enveloped virion with an icosahedral nucleocapsid with glycosylated membrane proteins.

EPIDEMIOLOGY

Although eradicated from many countries, CSF continues to cause serious problems in different parts of the world [2]. In South East Asia particularly in Indonesia, Korea, Malaysia, Myanmar, Mongolia, Philippines, Taiwan and Vietnam the disease is prevalent. The situation in most of Africa is uncertain, but the disease is not reported to be a problem, except in Madagascar. But recurrent sporadic outbreak has been reported from South Africa [3]. As of 2005; Australia, Canada, the Great Britain, Iceland, Ireland, New Zealand, Scandinavian Countries and USA are reported to be free from CSF. The disease is enzootic in most of the pig producing states [4-5] and particularly in the North Eastern states of India [6] and is considered to be the major constraint for the growth of piggery in the North Eastern states. Not only the outbreak of CSF, but also the occurrence of CSF virus in the tissues of pigs has been reported from different states of India [7]. Three defined fragments of the genome *viz.,* 5´ NTR, E2 and NS5B were selected and a standardized protocol for the calculation of phylogenetic trees including the nomenclature for the genetic types were established. The isolates analyzed, showed that they could be assigned into one of three main genetic groups (1, 2 and 3). Most historic isolates from Europe and America and some Asian isolates were found in one of these subgroups. Most recent isolates from different regions in Europe and several from Asia were in group 2 and they were again subgrouped into 2.1, 2.2 and 2.3 respectively. Finally, viruses from Asia fell into one of the sub-groups in group 3. The three viral subgroups (3.2, 3.3 and 3.4) were from Korea, Thailand and Japan/Taiwan, respectively. A single virus isolated in the United Kingdom in the 1960s constitutes Group 3.1 [8.] Even though group 1 viruses are predominant in India, but there is also reports of rapid spread of group 2 viruses in the country [9].

GENOMIC ORGANIZATION OF CSFV

The genome of CSFV is a single stranded RNA of positive sense, approximately 12,300 nucleotides in length and contains a single large Open Reading Frame (ORF). The ORF is translated into a polyprotein that is processed into a mature protein by viral and host cell proteases [10]. The envelop contains three glycoproteins *viz.,* E1, E2 and a ribonuclease E^{rns} [11]. The ORF is flanked by a 5´ and 3´ NTR. The large ORF encodes a single polyprotein which is co and post translationally cleaved into the proteins N-Terminal protease (N^{pro}), Capsid protein C, three envelop glycoproteins *viz.,* E^{rns}, E1, E2, p7 and the non-structural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B. The NTR at the 5´end harbours an Internal Ribosomal Entry Site (IRES). Therefore, the RNA can directly be translated upon uncoating without the need of a cap structure. E2 is the immunogenic gene and induces high virus neutralizing titers of serum antibodies [12].

Despite close similarities to BVDV and BDV, CSFV form a distinct group that can be differentiated serologically or on the basis of genetic similarities [13]. The severity of the disease varies with the viral strain; while some strains cause acute disease with high mortality rates, others can result in mild or even sub clinical disease. The morbidity and mortality rates are high during acute infections and the case fatality rate can approach 100 per cent. Morbidity and mortality are lower in sub acute disease. Chronic infections are always fatal, but may affect only a few animals in a herd. The age and immune status of

the animals also affects the course of the disease, with lower mortality rates in adult pigs than younger animals. Classical swine fever should be suspected in pigs with signs of septicemia and a high fever, particularly if uncooked scraps have been fed, unusual biological products have been used, or new animals have been introduced into the herd. This disease may also be considered in herds with other symptoms, including breeding herds with poor reproductive performance and disease in piglets. It can be difficult to differentiate classical swine fever from other diseases without laboratory testing like virus isolation and molecular characterization. Further, the improvement of molecular tools for the detection of CSF virus has always been a subject of research as these tools play an important role in both eradication programmes and in experimental work [11]. The devastating economical impact of the disease on the pig industry has always been a stimulus for research. However, like the other pestiviruses, CSFV was a difficult virus to work with and major progress only became possible with the development and the availability of sophisticated virological methods especially during the last 15 years [14].

THE USEFULNESS OF LABORATORY METHODS FOR DIAGNOSIS OF CLASSICAL SWINE FLU FEVER (CSF)

The highly variable clinical picture of CSF often precludes a diagnosis on clinical and pathological grounds alone. Other diseases such as Salmonellosis, Pasteurellosis, Actinobacillosis and *Haemophilus suis* infections may be confused with acute CSF. Laboratory methods are therefore essential for an unambiguous diagnosis. Detection of virus or viral nucleic acid in whole blood and of antibodies in serum are the methods of choice for diagnosing CSF in live pigs, whereas detection of virus, viral nucleic acid or antigen in organ samples is most suitable in dead animals.

Diagnosis of CSF can be attained by

1. Based on clinical signs (Presumptive Diagnosis)

   The disease can spread in all age group of animals and the prevailing signs are pyrexia, huddling, inappetance, dullness, weakness, conjunctivitis, constipation followed by diarrhea and an unsteady gait. There may be purple discoloration of the ears, abdomen and inner thighs several days after the onset of clinical signs. In acute cases, animals may die within 1-3 weeks. Under certain circumstances, depending upon the age and condition of the animal and strain of the virus involved, sub acute or chronic illness may develop leading to stunted growth, anorexia, intermittent pyrexia and diarrhea. Congenital persistent infection may go undetected for months and may be confined to only a few piglets in the herd or may affect larger numbers leading to wasting in the absence of pyrexia. Chronic, persistent infections always lead to the death of the animal. As CSF virus affects the immune system, immunosuppression may develop leading to concurrent bacterial infections.

   PM findings:

   In acute cases, gross pathological lesions might be inconspicuous or absent. In typical cases, the lymph nodes may be swollen and marbled red and haemorrhages may occur on serosal and mucosal membranes of the intestinal organs. There may be petechial haemorrhages on the surface of kidney giving a ‘turkey egg’ appearance. Splenic infarctions may occur. In sub acute and chronic cases, necrotic or ‘button’ ulcers may

be observed in the mucosa of the gastrointestinal tract, epiglottis and larynx, in addition to the lesions observed in acute cases.

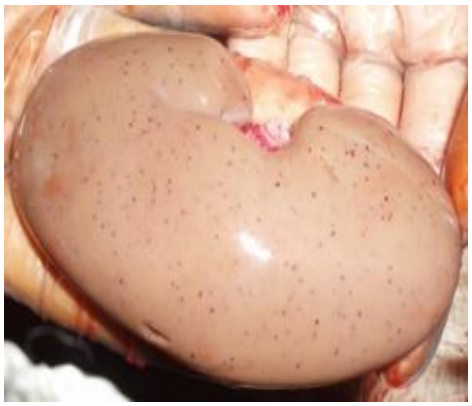

Figure 1.'Turkey egg' appearance of the kidney showing hemorrhages

Figure 2. Mesenteric lymph node

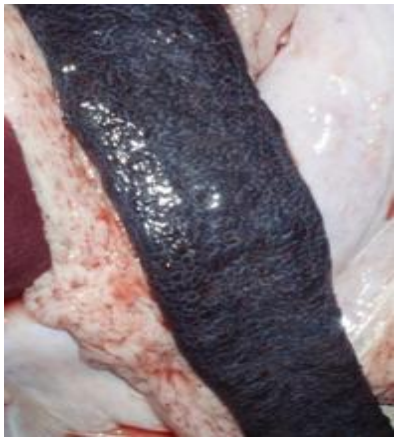

Figure 3. Spleen showing initial stage of infarction

2. Laboratory Diagnostic methods used for confirmatory diagnosis [15] can be listed as:

Identification of the agent

a) Immunological methods
   i) Fluorescent antibody test (FAT)
   ii) Immunoperoxidase procedure for differentiation of pestiviruses by monoclonal antibodies
   iii) Antigen-capture assay
b) Isolation of the virus or viral RNA
   i) Isolation in pig kidney (PK-15) cell line
   ii) Reverse transcription polymerase chain reaction (RT-PCR) for detection of viral RNA

Pen side diagnostic approaches
   i) Lateral flow
   ii) Magnetic bead microaaray

iii) Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP)
iv) Biosensor

Serological tests for international trade

i) Neutralising peroxidase-linked assay (NPLA)
ii) Enzyme-linked immunosorbent assay (ELISA)

Materials to be collected for laboratory diagnosis

As pyrexia is one of the first clinical signs of CSF and is accompanied by a viraemia, whole blood in heparin or ethylene diamine tetra-acetic acid (EDTA) or tissue materials can be collected from a few febrile animals for detection of virus or vial nucleic acid. This is more necessary in view of the serious consequences of an outbreak of CSF for trade in pigs and pig products. Random sampling is unsuitable for CSF diagnosis. But whole blood samples can be collected from a larger group of pigs for virus detection and reverse-transcription polymerase chain reaction (RT-PCR) analyses. During the first stage of the infection, tonsillar tissue is the most suitable, as this is the first to become affected by the virus irrespective of the route of infection [16]. In sub acute and chronic cases, the distal portion of the ileum may be the only tissue to give positive result when subjected to Fluorescent Antibody Test (FAT). The other tissue materials of choice at post mortem are pieces of kidney and lymph node.

i) Fluorescent antibody test (FAT)

The fluorescent antibody test (FAT) is used to detect CSFV antigen in cryostat sections of tonsils, spleen, kidney, lymph nodes or distal portions of the ileum. Tissues should be collected from several (febrile and/or diseased) animals [17] and transported without preservatives under cool conditions, but not frozen. Cryostat sections are stained directly with anti-CSF immunoglobulin conjugated to fluorescein isothiocyanate (FITC) or indirectly using a secondary FITC conjugate and examined by fluorescence microscopy. A negative FAT result does not completely rule out CSF infection. When suspicion of CSF continues, further samples should be obtained or attempts should be made to isolate the virus in cell culture (e.g. pig kidney [PK-15]) or any other cell line of pig origin known to be free from *Pestivirus* contamination.

The FAT involves the use of an anti-CSF immunoglobulin prepared from a polyclonal antibody to CSFV that will not distinguish between the antigens of different pestiviruses. Conjugates used for the FAT on cryostat sections or inoculated cell cultures should be prepared from anti-CSFV gamma-globulins raised in specific pathogen free pigs. The working dilution of the conjugates (at least 1:30) should combine a maximum brilliance with a minimum of background. The test should only be performed on samples from fresh carcass as autolysis and bacterial contamination can often result in high background staining.

FAT is almost routinely used in some diagnostic laboratories as it provides quick and inexpensive presumptive diagnosis. However, the quality of the primary antibody is critical to obtain accurate results. So these reagents should be fully characterized for their specificity and sensitivity. False-negative results may appear if the cut tissue section does not have any infected cells. A shortage of highly trained technicians, along with

development of newer techniques like PCR and ELISA has decreased the testing in some diagnostic laboratories [18].

ii) Immunoperoxidase procedure for differentiation of pestiviruses by monoclonal antibodies

The use of a panel of three monoclonal antibodies (MAbs), either horseradish peroxidase (HRPO) or FITC conjugated, or used in conjunction with an anti-mouse conjugate and specifically detecting all field strains of CSFV, vaccine strains of CSFV and ruminant pestiviruses respectively. A prerequisite is that the MAb against CSFV recognizes all field strains and that the anti-vaccine MAb recognizes all vaccine strains used in the country. No single MAb selectively reacts with all ruminant pestiviruses [19]. The use of MAb to differentiate a CSF vaccine strain can be omitted in nonvaccination areas. A polyclonal anti-CSF immunoglobulin conjugated to HRPO serves as a positive control. Caution should be exercised when using evidence of a single MAb as sole confirmation of an isolate as CSF. This test allows an unambiguous differentiation between field and vaccine strains of CSFV on the one hand, and between CSFV and other pestiviruses on the other [20].

iii) Antigen-capture assay

Antigen-capture enzyme-linked immunosorbent assays (ELISAs) are rapid tests for diagnosis of CSF in live pigs. These tests are useful for screening herds suspected of recently infection (advantages). The ELISAs are of the double-antibody sandwich type, using monoclonal and/or polyclonal antibodies against a variety of viral proteins in either serum, the blood leukocyte fraction or anticoagulated whole blood; in addition, some test kits can be used to test clarified tissue homogenates [21]. The technique is relatively simple to perform, does not require tissue culture facilities, is suitable for automation and can provide results within half a day. The main disadvantage of these tests is that they are less sensitive than virus isolation, especially in adult pigs and mild or sub clinical cases. Moreover the lowered specificity of these tests should also be taken into consideration.

iv) Isolation of virus

Isolation of CSFV is best performed in rapidly dividing PK-15 cells seeded on to cover-slips simultaneously with a 2% suspension of the tonsil in growth medium. Other pig cell lines may be used, but should be demonstrably at least as sensitive as PK-15 cells for isolation of CSFV. The cultures are examined for fluorescent foci by FAT after 24–72 hours or after 4–5 days incubation are fixed for immunoperoxidase staining. Isolation of virus in cell cultures is a more sensitive but slower method for diagnosis of CSF than immunofluorescence on frozen sections.

v) Reverse transcription polymerase chain reaction (RT-PCR) for detection of viral RNA

Many methods for RT-PCR have been described and are still being developed [22]. Due to its speed and sensitivity, RT-PCR is a suitable approach for screening suspected cases of the disease [23]. The molecular epidemiology of CSF is based on the comparison of genetic differences between virus isolates. RT-PCR amplification of CSFV RNA followed by nucleotide sequencing and subsequent phylogenetic analysis are the simplest methods to make these comparisons. The three defined fragments of the genome *viz.*, 5´ NTR, E2 and NS5B are used for standardizing protocol for the calculation of phylogenetic trees including the nomenclature for the genetic types [8].

Oligonucleotide primers for amplification of 5´ NTR gene of CSFV [24]

CSFV – UP1 (sense): 5´ CTA GCC ATG CCC WYA GTA GG 3
CSFV – UP2 (antisense): 5´ CAG CTT CAR YGT TGA TTG T 3´

However, it has to be kept in mind that false positive as well as false negative results can occur during RT-PCR due to laboratory contamination and inhibitors respectively.

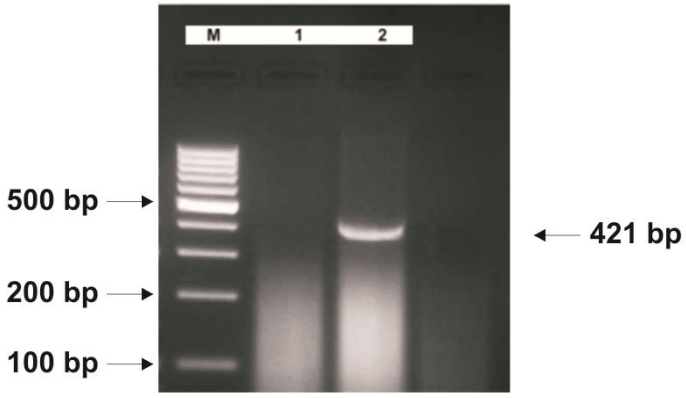

Figure 4. Agarose gel electrophoresis of PCR amplified product of 5´ NTR gene of CSFV from cell culture fluid. Lane M – 100 bp DNA ladder, Lane 1 – Negative Control, Lane 2 – Amplified 5´ NTR gene of CSFV

This internationally accepted method is rapid, specific and more sensitive than antigen-capture ELISAs or virus isolation making it particularly suitable for preclinical diagnosis [25].

A brief account of the pen side tests for the diagnosis of CSF are given in the subsequent paragraphs. These tests are less studied but are of great diagnostic importance.

i) Lateral flow

Antigen detection ELISA may utilize various assay formats including traditional ELISA plate formats or lateral flow devices, often called immunochromatographic strips. Lateral flow devices typically use a solid phase membrane with test and control lines coupled with absorbent pads. The strips may be placed into a test sample or the sample may be added to a designated area of the strip. These test formats may be used with sera or whole blood samples or tissue homogenates depending on the localization of the virus available processing method and quantity of target antigen in a given specimen. Advantages of lateral flow assays are that they are generally rapid, simple to perform and require minimum laboratory infrastructure relative to molecular techniques. However assay sensitivity may present challenges for the detection of the virus and timing of sample collection may be critical. The antigen must be present in adequate quantity to allow direct detection by these methods and appropriate high quality antisera or monoclonal antibody-based reagents must be available [18].

ii) Magnetic bead microarray

It is a novel method for the rapid detection and identification of the recognized pestiviruses including CSFV. The analysis of pestivirus PCR products is performed on microarrays by means of magnetic bead detection. The process utilizes an oligonucleotide array, onto which 5′ biotinylated PCR products are hybridized, followed by visualization with streptavidin-coated magnetic particles by the naked eye, microscope or biochip reader. This test is highly sensitive, specific and detection procedure is simple because of

which it provides a powerful tool for detection and identification of the pathogen. Considering the simplicity of the assay, the protocols for hybridization and magnetic bead detection offer an emerging application for molecular diagnosis that is amenable for use in a modestly equipped laboratory [26].

iii) Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP)
This method is useful for rapid pre-clinical detection of CSFV. RT-LAMP can be finished within one hour using a set of 4-6 primers targeting the 5' NTR is used under isothermal condition at 65° C [27-28]. The assay has higher sensitivity than that of RT-PCR with a detection limit of 5 copies per reaction and may range upto 100 copies. The greatest advantage is that there is no chance of cross-reactivity from the samples of other related viruses viz., porcine circo virus type 2, porcine parvo virus, Pseudorabies and Japanese encephalitis. This shows that the CSFV RT-LAMP assay is a valuable tool for its rapid, cost-effective detection [27].

iv) Biosensor
It comprises of a handheld microelectromechanical (MEMS) Point Detection Sensor employing a magnetic bead-based assay for the diagnosis of CSF. The sensor contains bivalent recombinant antibody molecules specific for the E2 and Erns for capture of CSFV from tissue and fecal/manure samples. This is a sufficiently cheap pen side test that can be used for extensive surveillance and is useful for risk management of CSFV including biosecurity measures at the farm level [29.]

The prescribed tests for international trade of animals are as follows:
i) Neutralising peroxidase-linked assay (NPLA)
The prerequisite for NPLA test is inactivation of sera at 56°C for 30 minutes. Microtitre plate is used for carrying out the NPLA test. For international trade purposes, it is best to test the serum with an initial dilution of 1/5 (1/10 final dilution). Appropriate controls to ensure specificity and sensitivity of reactions are to be incorporated into each test.

ii) Enzyme-linked immunosorbent assay (ELISA)
Competitive, blocking and indirect techniques may be used [30]. Care should be taken to minimize cross-reactions with BVDV and other pestiviruses. The antigen used for ELISA should be derived from or correspond to viral proteins of one of the recommended CSFV strains. Cells used to prepare antigen must be free from any other *Pestivirus* infection. Polyclonal antisera for competitive or blocking assays should be raised in pigs or rabbits by infection with one of the recommended CSFV strains or with the lapinised C strain. MAbs should be directed against or correspond to an immunodominant viral protein of CSFV. Indirect assays use an antiporcine immunoglobulin reagent that detects both IgG and IgM.
The sensitivity of the ELISA needs to be high enough to detect positivity of any serum from convalescent animals, i.e. at least 21 days post–inoculation that reacts in the neutralisation test [31]. The ELISA may only be used with serum or plasma samples derived from individual pigs. If the ELISA procedure used is not CSF-specific, then positive samples should be further examined by differential tests to distinguish between CSF and other pestiviruses. Recently, a novel ELISA has been described that uses fused

protein derived from viral peptides [32-33]. There are claims regarding the greater sensitivity and earlier detection of antibody by this ELISA.
The immunoassays for international trade are in general quick and inexpensive. But the reagents need to be fully characterized to increase sensitivity and specificity.

Differentiation of infected from vaccinated animals

The E2 glycoprotein in a purified form is capable of inducing a protective immunity [34]. This forms the basis for the development of E2 subunit vaccine that only the E2 glycoprotein. The E2 glycoprotein is produced in cultures of insect cells infected with the baculovirus vector [35]. Pigs vaccinated with a sub-unit marker vaccine only develop antibodies against the E2 glycoprotein, whereas pigs that are naturally infected develop antibodies against different viral proteins (e.g. E2, E^{rns}, NS3). Consequently, it is possible to distinguish between an infected and a vaccinated pig by means of an ELISA test that only detects antibodies against the E^{rns} glycoproteins [36]. Currently, there are two E2 sub-unit marker vaccines commercially available and both are licensed via a European procedure. Also two similar differential diagnostic antibody ELISA tests are available [37].

CONCLUSION

Classical Swine Fever Virus (CSFV) is an important veterinary pathogen which can cause widespread epidemics. With the critical need for improved diagnostic tests to detect viral infection, efforts need to be concentrated on the development of simple, rapid, noninvasive tests that can be performed without expensive laboratory equipment. In this context even though various molecular tools are very promising, but there is need to search for more rapid and accurate tests as well as an earlier detection system in preclinical state.

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