MOLECULAR CHARACTERIZATION OF CLASSICAL SWINE FEVER VIRUS VACCINE STRAIN BY PARTIAL GENOME SEQUENCING AND ANALYSIS

M.Sc. Thesis

(2019)

Submitted to

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Tribhuvan University

Kirtipur, Kathmandu, Nepal

For partial fulfillment of the requirement of the

M Sc. degree in Biotechnology

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Date: **May15, 2019**

RECOMMENDATION

This is to certify that the research work entitled "**MOLECULAR CHARACTERIZATION OF CLASSICAL SWINE FEVER VIRUS VACCINE STRAIN BY PARTIAL GENOME SEQUENCING AND ANALYSIS"** has been carried out by **Mr. Tika Bahadur Budha** under my supervision.

This thesis work was performed for the partial fulfillment of the Master of Science in Biotechnology under the course code BT 621. The result presented here is his/her original findings. I/we, hereby, recommend this thesis for final evaluation.

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Certificate of Evaluation

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Glossary Acronyms

BD	Border Disease
BDV	Border Disease Virus
BVD	Bovine Viral Diarrhoea
cDNA	Complementary Deoxyribo Nucleic Acid
Concn.	Concentration
CSF	Classical swine fever
CSFV	Classical swine fever virus
Ct	Threshold cycle
Cq	quantification cycle
DNA	Deoxyribo Nucleic Acid
dNTPs	deoxyribonucleoside Triphosphates
ds	Double stranded
EDTA	Ethylene Diamine Tetra acetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
et al.,	*et alia*
EU	European Union
F	Forward
FAT	Fluorescent Antibody Tests
Fig.	Figure
gm	gram
i.e.	idest (that is)
IPT	immuneperoxidase test
M	Molar
Ml	Millilitre
Nm	Nanometre
Npro	N-terminal auto-proteinase
NS5B	Non structural 5B
nt	Nucleotide
NTR	Nontranslated Region
OIE	Office international *des* epizootics / World Organisation for Animal Health
ORF	Open reading frame
PCR	Polymerase Chain Reaction
PK -15	Pig Kidney cell line
R	Reverse
RdRp	RNA-dependent RNA polymerase

RNA	Ribo Nucleic Acid
rpm	revolution per minute
rRT-PCR	real time Reverse Transcription Polymerase Chain Reaction
RT –PCR	Reverse Transcriptase - Polymerase Chain Reaction
Sec	Seconds
TAE	Tris acetate EDTA
Taq	*Thermusaquaticus*
Temp.	Temperature
Tm	Melting temperature
UTR	Untranslated region
UV	Ultra Violet
V	Volt
VI	Virus Isolation
VNT	Virus Neutralization Test

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ABSTRACT

Molecular characterization of classical swine fever virus vaccine strain by partial genome sequencing and analysis.

Classical swine fever (CSF), a listed disease of the Office International des Epizooties (OIE), is an important, highly contagious and often fatal pig disease with widespread economic implications. The classical swine fever virus (CSFV) is the causative agent of this disease which is closely related to the other members of the genus *Pestivirus*. Classical swine fever (CSF) causes major losses in pig farming, as it has various degrees of disease severity. Therefore the endemic countries are applying the strategy of efficient live attenuated vaccines against classical swine fever virus (CSFV). However, despite of such intensive vaccination programs in these areas for more than 20 years, CSF has not been eradicated. So the vaccination is being important to prevent the loss from CSF disease in pig farming. The aim of this work was to detect CSFV in the vaccine developed and molecular characterization of the virus by using molecular techniques. In this study, four live attenuated vaccine samples; three from Hester Biosciences Nepal Private Limited and one from Central Biological Production Laboratory were analyzed. The 5'Non Translated Region (5'NTR) and Envelope Glycoprotein (E2) gene segments of all the vaccine samples were successfully amplified with required band size of 271 bp by using the Reverse Transcription nested Polymerase Chain Reaction(RT-nPCR) technique. From the amplified PCR product, nucleotide sequences of E2 and 5'NTR were determined which were used for sequence alignment and phylogenetic analysis. The phylogenetic analysis based on E2 segments indicated 97-99% and identities at the nucleotide level with other CSFV strains databases available in the NCBI. Furthermore, the 5'NTR showed 83-98% nucleotide similarity with the reference sequences. From the phylogenetic analysis based on both the gene segments it was found that all the tested vaccine samples were found closer to other reported lapinized attenuated vaccine strains namely Chinese strain, Lapinized Philippines Coronel (LPC), hog cholera lapinized virus (HCLV) and Riems C strains. As these results showed the tested vaccine strains are closest to other reference vaccine strains of subgroup 1.1, we concluded that these strains could be grouped into subgroup 1.1. In addition, the SYBR green based RT-qPCR also showed that all the tested samples were CSFV positive and also indicated that the seed vaccine that was used as positive control constitute the highest number of viruses. To our best of knowledge, this is the first report in Nepal for molecular characterization of CSF vaccine strain.

Key words: 5'NTR, E2, CSF, CSFV, Lapinized attenuated vaccines, NS5B, Phylogenetic analysis, RT-nPCR, RT-qPCR, Sequencing

Chapter I

INTRODUCTION

1.1 Background

Classical swine fever (CSF) is a contagious viral disease of domestic pigs and wild boar with high mortality rate. The disease, also known as hog cholera is caused by the classical swine fever virus which is a member of genus *Pestivirus* and *Flaviviridae* family and is a single serotype of classical swine fever virus(CSFV)(OIE, 2014). The disease has huge negative impact on animal health and pig industries and is therefore notifiable to the World Organization for Animal Health (OIE). It is classified as an OIE List A disease, i.e. all the suspected cases of CSF disease have to be investigated strictly and the confirmed outbreak has to be notified(Volker Moennig, 2000). The main route of infection in field cases is oronasal and the disease is highly transmitted from one pig population to another either by contact between live pigs or by feeding the contaminated pig meat to the healthy one (Zhang, Cao, Wu, & Cui, 2011).

The clinical sign and symptoms are similar in domestic pigs and wild boar which can be seen after an incubation period of four to seven (seldom 10) days after the infection with the CSFV. The progression of the disease is highly dependent on type of virulence strain, host responses and different secondary infections. The predominant sign and symptoms of the disease in all age groups are pyrexia, loss of appetite, inflammation of conjunctiva, dullness and weakness in animal, huddling and constipation followed by diarrhea(OIE, 2014). There are different courses of disease i.e. an acute, a chronic and a persistent course. The acute form is transient or lethal one and the persistent case requires infection in animal during pregnancy(Blome, Staubach, Henke, Carlson, & Beer, 2017).

In most of the parts of the world with significant pig production, there is sporadic presence of the CSF disease. Several countries of South and Central America, some parts of Eastern Europe and their neighboring countries, as well as some Asian countries including India are known as endemic of the disease whereas there is little known about the African countries(Blome et al., 2017). Some countries, including Australia, Canada, New Zealand, United States of America, and some Member States of the European Union have been succeeded in eradicating CSF after implementation of strict control measures(Volker Moennig, 2000).

1.2 A brief history

There is no any historical record that clearly indicates where the CSF disease was originated. It is supposed that the first record of the disease was in 1833 in Ohio, USA. Whereas Hanson (1957) proved that this claim was not sustainable from original evidence as the Ohio outbreak was only one among many in the USA in the early 19th century; indeed, an epizootic was reported from France in 1822 that resembled to CSF disease. This indicated the possibility of introduction of the disease to America with pigs from Europe. The disease was then widespread in Europe and America by the 1860s which might be facilitated by the development of railways during the mid -19th century (Edwards et al., 2000).

To prevent the devastating loss from the disease, immunization strategy was employed for controlling the disease. The host animals were immunized with different ways such as by simultaneous use of antiserum and virulent virus, crystal violet inactivated vaccine, attenuated live virus vaccine in the 19th century. There was also development of use of immunological techniques such as ELISA and molecular techniques for diagnosis purposes in the 19th century after recognition of its relationship with bovine viral diarrhea virus(Edwards et al., 2000).

1.3 Classical swine fever virus

1.3.1 Systematic position

Domain:	Virus
Unknown:	"Positive sense ssRNA viruses"
Unknown:	"RNA viruses"
Order:	Nidovirales
Family:	*Flaviviridae*
Genus:	*Pestivirus*
Species:	Classical swine fever virus
	(CABI, 2018)

1.4 Clinical spectrum of CSF disease

The clinical signs of the disease vary with the virulence of the virus, age and immune status of the host animal. In older breeding of pig, the infection is mild or sub-clinical. There are different forms of the disease mainly base on the age, breed and immune status of the host animal.

a. Acute form

This is the most severe form of the disease which is mostly seen in piglets of up to 12 weeks of age. The initial symptoms of this form are high fever i.e. 40◦C, whereas in the case of adults the temperature may not exceed 39.5◦C, loss of appetite, drowsiness, conjunctivitis, enlarged and discolored lymph nodes, respiratory signs and constipation followed by diarrhea. Neurological signs like staggering gait with weakness of hind limbs, incoordination of movement and convulsions are also seen. After the second and third week of infection, a typical haemorrhages on the skin of ear, tail, abdomen and the inner side of the limbs is observed. On post mortem after the death of the infected animal, pathological changes such as swollen lymph nodes, spleen and kidneys, haemorrhagic lymph nodes, urinary bladder, larynx, epiglottis and heart can also be observed. There is also a chance of secondary infection in the infected animal as the CSF disease causes severe leucopenia and immunosuppression. The clinical signs are less specific and can be recovered by production of antibodies in increasing age of the infected pigs, the antibodies produced against CSFV become detectable after 2-3 weeks of exposure to virus.

b. Chronic form

The chronic form of CSF is fatal which usually occurs in animals lacking effective immune response against the infection. The initial signs and symptoms of this form are similar to that of the acute form but in later non-specific signs such as intermittent fever, chronic enteritis and wasting are seen. Animals may survive for 2-3 months after they got infection. Antibodies may be produced but not able to eliminate the virus as they get neutralized by virus. This form lacks pathological changes such as haemorrhages on organs and serosae but the necrotic and ulcerative lesions on the ileum, ileocaecal valve and rectum are common in animal with chronic diarrhea.

c. Congenital form

CSFV can cross the placenta of pregnant animals, so can infect fetuses during all stages of pregnancy. Infection during the early phase of pregnancy may result in abortions and stillbirths, mummification and malformations. A sow of 50-70 days of pregnancy if got infection with CSFV gives birth of a viraemic piglets with poor growth, wasting or occasionally congenital tremor and survive for several months. These piglets are the dangerous reservoir of virus and shed large amounts of virus thereby spreading the disease among the pig population.

1.5 Epidemiology and geographical distribution of CSF

1.5.1 Global distribution of CSF

The distribution of genotypes and sub-genotypes of CSFV shows distinct geographical pattern. All the European CSFV isolates that were isolated in 1990s and later belong to different sub-groups of group 2, which is the most prevalent genotype over the last decades on the global scale. The field isolates from the American continent are included in genotype 1. The isolates of sub-genotype 1.1 and 1.3 are from Argentina, brazil, Colombia, Mexico and Honduras, Guatemala respectively. The group 3 isolates are present solely in Asia. Whereas, in the case of Africa and the Middle East, the CSF case is little known with exceptions of 2005 outbreak in South Africa and the 2009 outbreak in Israel both of which were caused by 2.1 sub-genotypes.

In the context of Asia, there was found changes in the historical situation of the CSFV distribution. Reports from India showed that the sub-genotype 1.1, 2.1 and 2.2 are co-circulating which is different from the historical situation of dominance of group 3. In China there is presence of high variability of strains that consists of 1.1, 2.1 and 2.3 sub-genotypes. In Taiwan also the historical 3.4 strains are replaced mainly by the Chinese 2.1 strains and also with 1.1, 2.2 and 2.3 according to some reports. 2.1 And 2.2 strains are reported from Laos, 3.2 and 2.1 from Korea and 3 from Japan. In the case of Nepal sub-genotype 2.2 was reported(Postel, Jha, Schmeiser, & Becher, 2013).

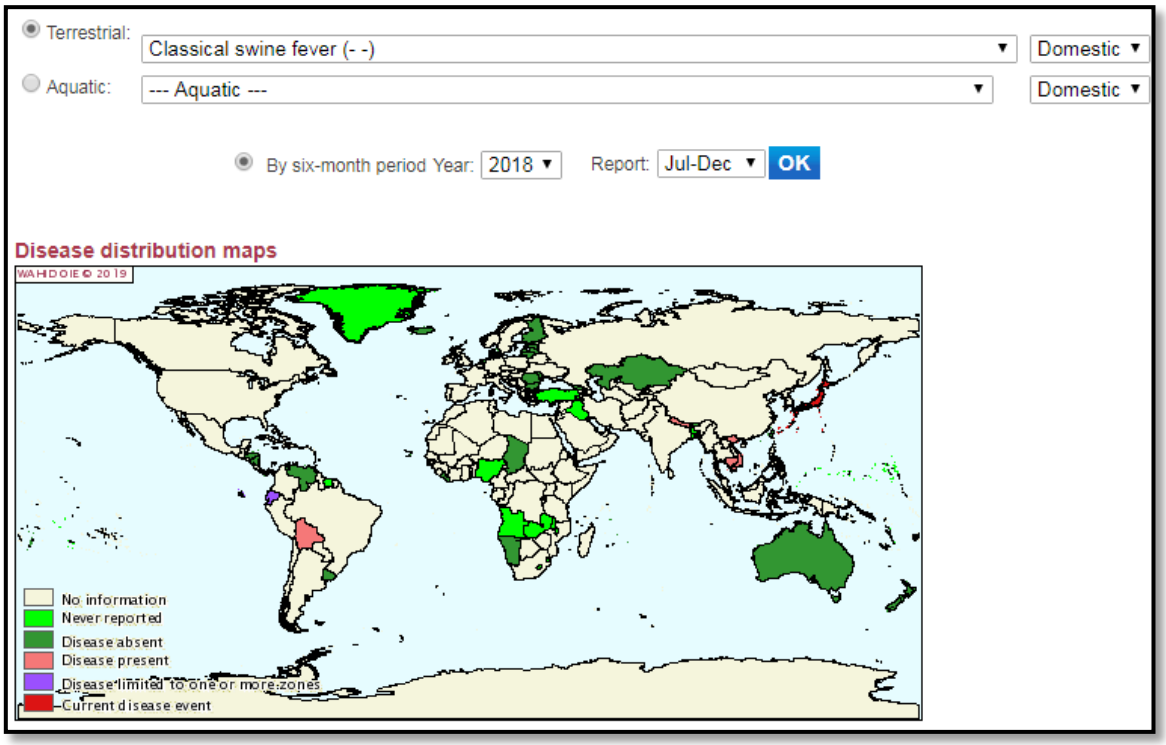


Figure 1. 1: Map showing the global distribution of CSF disease based on six-monthly (Jul-Dec,2018) reports submitted by the OIE member countries to OIE(Interface, 2019).

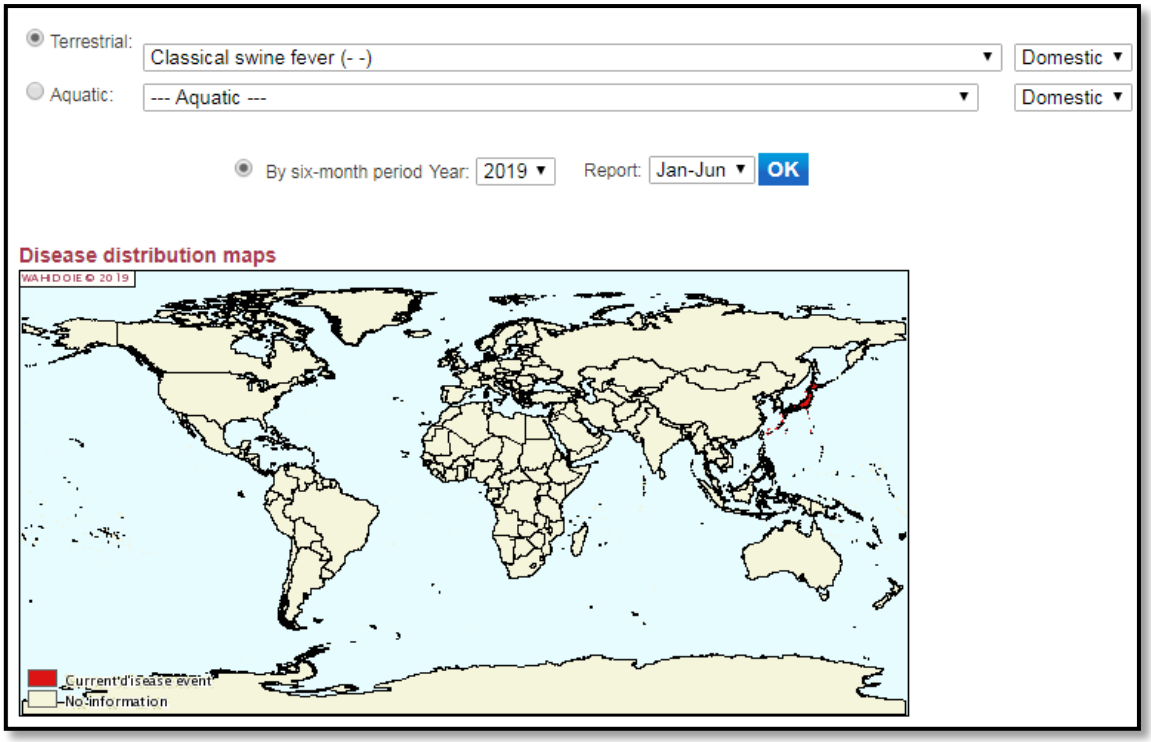


Figure 1. 2: Map showing presence or absence of CSF disease in the world based on six-monthly (Jan-Jun, 2019) reports submitted by OIE member countries to the OIE(Interface, 2019).

1.5.2 Scenario of CSF epidemiology in Nepal

The livestock sector is an integral component of farming system that contributes an important role in economy of Nepal as it is a source of food, income and employment. Animal husbandry, dairy and fisheries are the major sectors of livestock in Nepal which play important role in socio-economic development of the country. The demand of livestock and livestock products such as meat, milk, and poultry is continuously increasing with rapidly increasing human population. It is an important sector in the context of Nepal as major portion of the country is composed of rural villages. It plays a crucial role in reduction of rural poverty. Among others, pig farming is a major part of the livestock sector for meat production in Nepal. Pig is much preferred among other meat producing animals as it has short generation interval, faster growth rate and efficiency of feed conversion. As per 2017 census, the population of pig in Nepal is 1328036 and the meat (pork) production is 24535 Mt.(Department of lovestock services, 2016/17). However the pig industry in the country has been facing different challenges in the growth and reproduction of pigs. One of the factor that negatively impact on the sustainability of the pig industry is infectious diseases, among them CSF is one of the major constraints to pig farming. There were total of 153 CSF outbreaks were reported from different districts of Nepal during 2005-2019 which were confirmed based on clinical symptoms and postmortem examination(Interface, 2019).

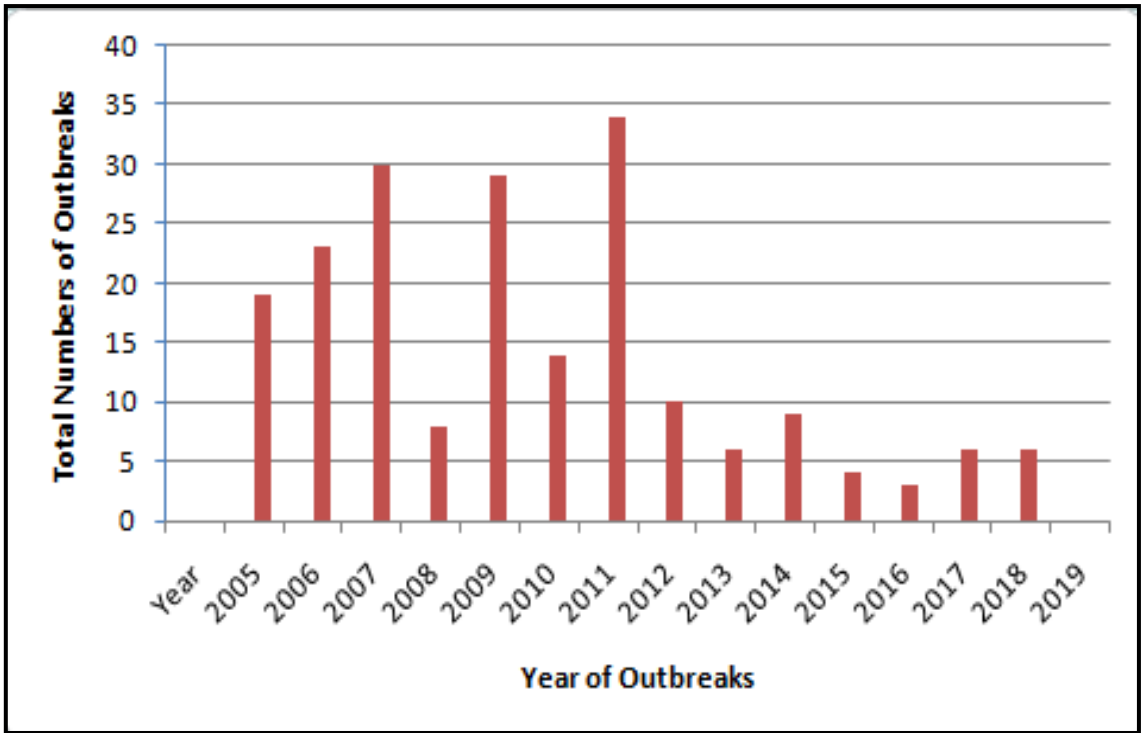


Figure 1. 3: The situation of CSF in the country on yearly basis from 2005-2019 AD(Interface, 2019)

1.5.3 Transmission of disease

The main route of transmission of disease is oronasal. The causative virus spread from infected pig to healthy one easily by direct or indirect contact with infected pig or by consumption of feed contaminated with the virus(V Moennig et al., 2003). The disease might also transmit upon contact with conjunctiva, mucus membranes, skin abrasions, insemination, and the use of contaminated instruments. The infected pigs have high titer viremia which continuously shed the virus out until the death of animal or specific antibodies have developed. The main excretion routs are by saliva, lacrimal secretions, urine, feces, and semen. Transmission of the disease from pregnant sows to fetuses at all the stages of gestation is also possible which result the persistently infected offspring. The wild boars infected with CSFV can serve as the reservoir of the virus and may transmit the disease to domestic pigs(Blome et al., 2017).Over short distance, air-borne transmission of the disease among the animals is possible(Dewulf, Laevens, Mintiens, De Kruif, & Koenen, 2000).

1.5.4 Pathogenesis of virus and immune evasion strategies

The virus first attacks the lymphoid tissues after its entry through oronasal route and initial multiplication of the virus occurs in tonsil. Then the virus move to blood and spread to many other lymphoid tissues like lymph nodes, spleen, kidney and pancreas etc. The virus can also cross the placental barrier and occasionally infect the nervous tissues of brain(Shivaraj, 2014).

The virus causes detrimental effects on the immune and hematopoietic systems where its major targets are monocytes in peripheral blood and macrophages in the lymphoid organs(Summerfield, Knötig, & McCullough, 1998). Replication of virus occurs in monocytes and macrophages that induces the release of cytokines including prostaglandin-E2 and interleukin-1 that causes fever and haemorrhages in the host(Knoetig, Summerfield, Spagnuolo-Weaver, & McCullough, 1999). Peripheral blood mononuclear cell (PBMC) subpopulations such as CD4+, CD8+ and IgM+ lymphocytes are also sites where replication of the virus occurs(Lee, Wang, & Chien, 1999). During the infection there occurs depletion of both B and T lymphocytes in the circulatory system as well as lymphoid tissues and the neutralizing antibodies may not appear until three weeks after the infection. The first viral protein Npro is an autoprotease which limits type I interferon induction in various cells thereby avoiding the antiviral effects of type I interferon. The virus also causes the inhibition of nitric oxide production in infected macrophages(Shivaraj, 2014).

1.5.5 Prevention and control

Biosecurity: Biosecurity measures are important for the protection of animals at herd as well as to prevent the introduction of the disease in a country. Its importance is increasing day by day with increasing in density of pigs and industrialization of pork husbandry. Biosecurity is a multi-domain aspect which has to be implemented on several levels on a working farm. An adequate fencing and a gate blocking the entrance to the farm should be maintained to limit and restrict the direct access to the pig pens and the whole farm. Though human are not a host of the causative virus, they can act as a mechanical vector as they can be contaminated during hunting, visiting or working other farms with the disease. There should be available of clean clothes and separate clothing and changing room for workers to reduce the transmission of the disease. There should also be available of disinfectant baths at the entrance of the pens for boots/ shoes and the disinfectant baths should regularly be cleaned. Transmission of CSF by mechanical means is major as CSFV has a limited host range. Nevertheless, the programs that control the insect and rodent should be implemented and presence of other animals on the premises should be limited. Another risk issue is the transmission of CSFV by air or aerosols as CSFV survives aerosolisation and remains infectious at least for 30 minutes. Therefore aerosol produced during cleaning/disinfectecting should be minimized(Matthias Kramera, Andy Haegemanb, & Greiser-Wilked, 2009).

Vaccination: The infection with CSFV in the endemic countries can be controlled by the systemic vaccinations campaigns. Oral vaccination of wild boar also can help in lowering the incidence of CSF Whereas vaccination should not be allowed in the CSF free

countries rather should be highly alert to diagnosed new outbreaks(Van Oirschot, 2003). There have been developed many live attenuated CSF vaccines by traditional methods either by serial passage in rabbits (e.g. the Chinese or C-strain) or by passage in tissue culture (e.g. Thiverval strain). These vaccines produced are remarkably effective to control losses in disease endemic area and also have assisted in the eradication of the disease from many areas of the world on combining with culling of infected animals(Graham et al., 2012). There have been developed and tested different vaccination approaches for control of the disease in wild boar; some of them directly under field conditions (e.g. lyophilized vaccines in Russia), others under experimental conditions. In 1990's, kaden et al. (2000) developed and tested oral bait formulations to deliver the vaccines on a large scale and found to be satisfactory option for controlling CSFV in wild boar in Western Europe. However, upon implementation it was found that the process of vaccination and design need further improvement as adaptations were introduced in wild boar(Rossi et al., 2015). Though there is also another non-vaccination way of controlling CSF disease i.e. stamping-out policy; modified live vaccine (MLV) are found to be better as comparing to huge costs and inadequacy of stamping out and also the MLV can induce complete protection against virulent CSFV. Therefore, the MLV are still widely used as a control policy in the CSF endemic areas including Asia and central South America(Huang, Deng, Wang, Huang, & Chang, 2014). In Japan where MLV had used, no outbreak occurred between 1993 and 2003. In 2000, vaccination was suspended as the Government decided to prohibit the use of the vaccine without the authorization of local government. In 2004 five farms were confirmed with CSFV and the confirmed isolates were found different from the vaccine strain authorized for use in Japan and field virus previously isolated in Japan(Ozawa et al., 2006). In the case of Taiwan, no any cases of CSF have been reported in recent years as there have been used MLV as a control measure since 1950s(Huang, Deng, Wang, Huang, & Chang, 2014).

Nepal is also employing the strategy of vaccination against CSF disease for the prevention and control of the disease. The Central Biological Production Laboratory of Nepal is continuously producing a lapinized classical swine fever vaccine since it produced first in 1998(CBPL). In Nepal, the veterinary services including vaccination services are generally provided from District Livestock Service Office(PEAN, 2016).

1.6 Rationale of the Study

Nepal is an agricultural country with significant livestock population on other side. Pig farming constitutes the major part of the livestock with 1328036 pigs in the country in year 2016/17. Pig farming has not only played important role in improvement of the economy of the country but also played role in prevention of food scarcity by production of 24535 metric ton pork in year 2016/17(Department of lovestock services, 2016/17). Though there are many positive impacts of this on society and the nation, there are many challenges in pig farming; among them CSF is one of the most economically-damaging pandemic viral disease of pigs in the world. Classical swine fever (CSF) is most important contagious viral disease of pigs and wild boar and causes high mortality. Many governments take it very seriously and adopt strict control policies, which include compulsory vaccination for prevention, control and eradication of the disease. The rapid and reliable diagnosis of CSF is also very important for the implementation of control measures in time. As vaccination is the main strategy for prevention and eradication of CSF disease, the vaccine used should contain the correct virus strain and it should be free from other extraneous agents that might cause disease in the host. In this study, we aimed to check presence of required virus strain in the vaccine samples which will be used for vaccination of pigs against CSF disease. This work will assist in the implementation of the vaccine in pig farms for prevention of the CSF successfully. The data obtained from this study might also be useful in comparing the vaccines strains used in preparation of vaccine in Nepal with any other new vaccines if developed in country. Therefore, this study will assist in prevention of CSF disease in the country.

1.7 Research objectives

1.7.1 General Objectives:

Detection of classical swine fever virus in vaccine samples and their molecular characterization.

1.7.2 Specific Objectives:

1. Viral RNA detection in the vaccines
2. Confirmation of Classical Swine Fever by amplification of E2 and 5'NTR gene segments.
3. Molecular characterization and phylogenetic analysis of CSFV vaccine strains.
4. Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR) amplification of NS5B gene segment.

1.8 Research Hypothesis

1.8.1 Null Hypothesis

Ho 1 = There is absence of classical swine fever virus in the tested vaccine samples.

Ho 2 = The virus detected in the vaccine samples is different from the other reference vaccine strains in molecular level.

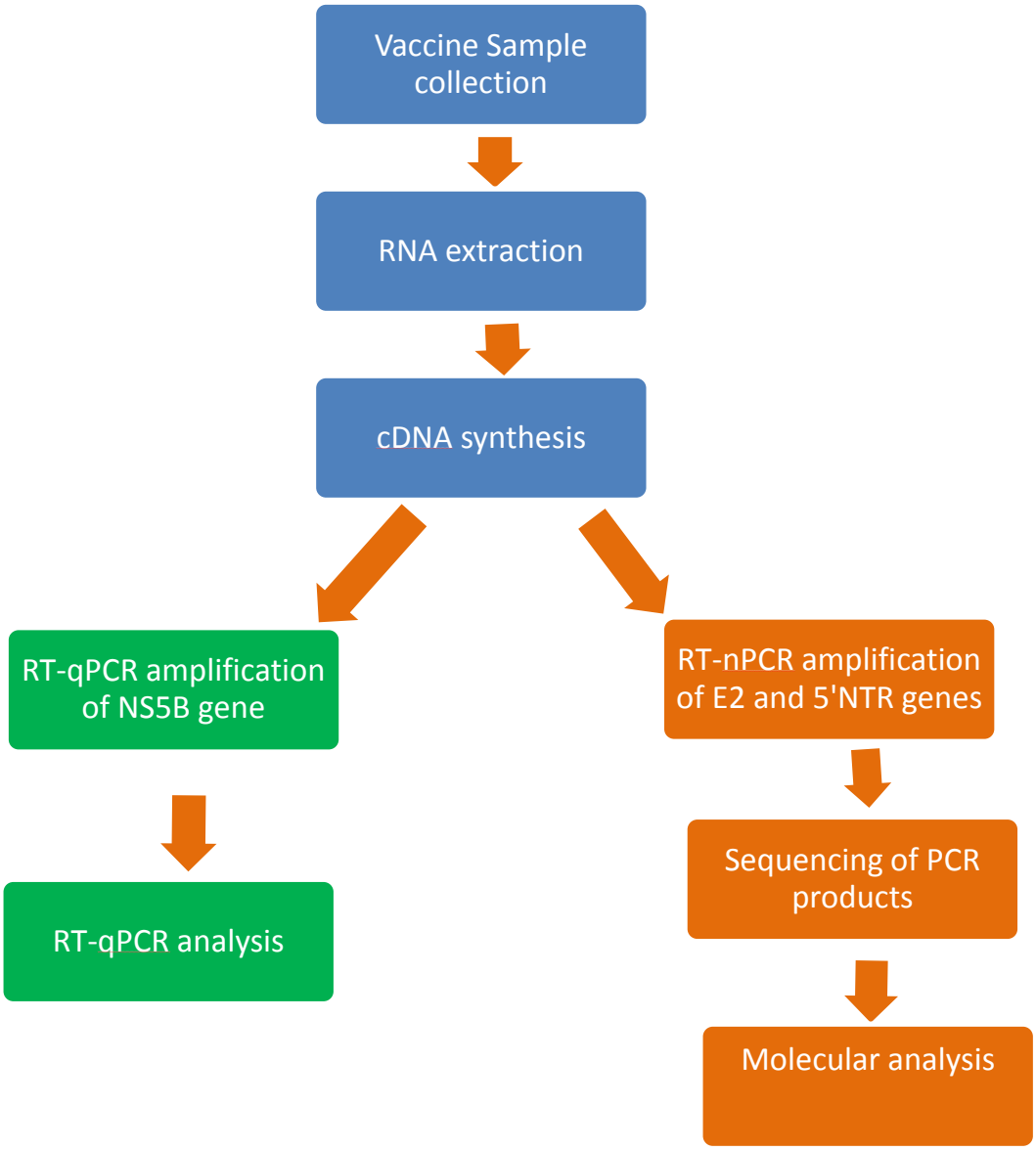
1.8.2 Alternative Hypothesis

H1 1 = There is presence of classical swine fever virus in the tested vaccine samples.

H1 2 = The virus detected in the vaccine samples is similar to other reference vaccine strains in molecular level.

1.9 Research Plan Design

With the view to run the research smoothly, flow chart was made to meet the claimed objectives.



Chapter II

Literature Review

2.1 Description of CSFV: Viral genome and its protein

CSFV is a member of family *Flaviviridae* and genus *Pestivirus* which is closely related to the bovine viral diarrhea (BVD) virus and the border disease (BD) virus of sheep. The virions are hexagonal shaped particles with about 30nm of electron-dense inner core. The inner core is surrounded by an spherical envelope of about 40-60 nm diameters(V Moennig, Floegel-Niesmann, & Greiser-Wilke, 2003). The genome of virus i.e. positive single stranded RNA comprises a single open reading frame (ORF) of length approximately 12.3 kb. The two ends of the viral genome is flanked by a 5'-nontranslated region (5'NTR) and 3'-nontranslated region (3'NTR) which encodes a single polyprotein with about 3898 amino acids. The single polyprotein is then processed into four structural (C, Erns, E1 and E2) and eight non- structural (Npro, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins with the help of viral and cellular enzymes(Zhang et al., 2011). The structural protein encoding genes are located towards the 5' end of the genome, whereas the genes encoding the non-structural proteins are found in the 3' two thirds of the genome (Paton et al., 2000).

2.1.1 Structural proteins

C protein: The C protein is a small core protein composed of many lysine and arginine amino acids. It has a protective function by forming a core protein- RNA complex inside the virion. It function not only as a structural protein but also as a gene expression regulator as it can activate the promoter of heat shock protein 70 gene and suppress the SV40 early promoter.

Erns protein: Erns (E0) glycoprotein is composed of 227 amino acids with an apparent size of 41-44 kDa and was formally termed gp44. Approximately half of the molecular mass of the mature Erns glycoprotein is made up of carbohydrate. It is associated with mature virions which involves in CSFV entry into the cell. The glycoprotein takes part in the initial attachment process of viral entry and in the post entry stages. The CSFV with Erns deletion make it non-transmissible. The Erns is considered to be the secondary glycoprotein for mediating CSFV infection as E2 is the major neutralizing antigen. Both Erns and E2 give protective immunity in the host as they induce viral neutralizing antibodies. The Erns is unique to Pestiviruses compared to other viruses in the family *Flaviviridae* as it is heavily glycosylated with N-linked glycans upto half of the apparent

molecular weight. It has also ribonuclease activity and its C-terminal domain controls translocation across eukaryotic cell membranes. N-glycan of Erns also play role in the blocking of IFN-β induction.

E1 protein: E1 protein is the smallest envelope protein with 33kDa molecular weight and 195 amino acids. It is a type I trans-membrane protein with an N-terminal ectodomain and a C- terminal hydrophobic anchor that is involved in viral adsorption to host cells. It contain highly conserved three putative glycosylation sites which are crucial to the virus cycle such as attachment to host cell receptors, entry, assembly of newly produced viral progeny and exit. Live-attenuated vaccines can be developed by modification of E1 glycosylation patterns.

E2 protein: E2 protein is the major envelope glycoprotein with molecular weight of about 51-55KDa consisting of 373 amino acids. It is exposed on the outer surface of the virion which is an important target for induction of the immune responses during the course of infection. There is presence of four antigenic domains (A, B, C, and D) located within the N-terminal half of the protein. It plays major role in virus attachment and entry by forming homodimers and heterodimer with E1. The processing of E2 is mediated by a host cell signalase which was concluded on the basis of amino acid sequence analysis and determination of the N-terminal. There was presence of typical hydrophobic signal sequences upstream of the E2 N-terminus within E1- coding sequences and a transmembrane anchor of about 40 hydrophobic amino acids at the C-terminus of E2. It has very important role in DNA vaccine development against CSF as it contains sequential neutralizing epitopes which are responsible for eliciting neutralizing antibodies.

2.1.2 Non-structural proteins

Npro protein: Npro (N-terminal auto-proteinase) protein is the first non-structural protein encoded in the ORF protein having 23KDa molecular weight and 168 amino acids. It is a cysteine proteinase having auto-protease activity for co-translational cleavage from the nascent downstream nucleocapsid ptrotein C and also has antagonistic effect on the IFN-α/β induction pathway. The cisteine proteinase has similarities to subtilisin-like proteinases which is not found in other viral systems. This protein is required for virulence of CSFV. After deletion of Npro, the mutants virus get attenuated and still having capacity to induce protective immunity in the pigs.

P7 protein: The P7 protein with 7KDa molecular weight and 70 amino acids lies on downstream of E2. It is a small hydrophobic protein flanked by signal peptidase

cleavage sites which requires for production of infectious virus but is not associated with the virus particles indicating it is not a major structural component of the virion. It forms the junction between the structural and the non-structural genes in Pestiviruses.

NS2 protein: NS2 is an auto-protease essential for the viral life cycle. It is associated with a cellular chaperone termed JIV (J-domain protein interacting with viral protein) that cleaves the NS2-3 protein between NS2 and NS3 which is required for the life cycle of *Pestivirus*. This protein also involves in inhibition of gene expression from different cellular promoters as well as in interference in cell proliferation.

NS3 protein: The NS3 protein is a multifunctional protein involved in the processing of the polyprotein. It possesses three enzyme activities require in virus replication. A serine protease stimulated nucleotide triphosphatase (NTPase) as well as RNA helicase activities which are located in the N-terminus of one-third of the protein and RNA and in the C-terminal portion respectively. Some evidence suggested that the protease and helicase/NTPase domains of the NS3 protein were functionally interdependent.

NS4A and NS4B proteins: NS4A acts as a cofactor for the serine protease activity for the formation of infectious particles as it is associated with the N-terminus of NS3. It plays an important role for the correct conformation, topology and functionality of NS3 within the infected cell. It also recruits other viral or cellular proteins such as NS4B and NS5A. A role in cytopathogenicity was associated with NS4B.

NS5A and NS5B proteins: NS5A is a phosphorylated protein and is an only protein of the replication complex that can be complemented in trans form. Its role in genome replication and translation has been proved as it was found to interacting with a subunit of the translation elongation factor 1A (TEF 1A). NS5B initiates replication by binding to the 3'-NTR of the viral RNA. NS5B is located at the extreme C-terminus of the polyprotein. NS5B contains motifs such as Gly-Asp-Asp (GAA) shared by RNA-dependent RNA polymerase (RdRp). The GAA motif is highly conserved among RdRps and has been demonstrated to possess RdRp activity. The RdRp is a key enzyme that initiates RNA replication by de novo mechanism without a primer and is a potential target for anti-virus therapy.

Non-translated region (NTR): The first entry site for viral replicases for initiation of RNA genome replication is the 3'-NTR of plus-strand RNA of CSFV genome. There is presence of secondary structure in the 3'-NTR which is unwind by helicase activity during

replication. It is mainly involved in the initiation of replication in the *pestiviral* genome but also play role in translation. The 3'-NTR is an important site for interaction between proteins and viral RNA genome and is essential for replicase and helicase activities. 5'-NTR contains the cis-elements required for the replication of the viral genome. It also has IRES for cap-independent translation initiation of the viral polyprotein which is co- and post- translationally processed by host cell and viral proteases(Zhang et al., 2011).

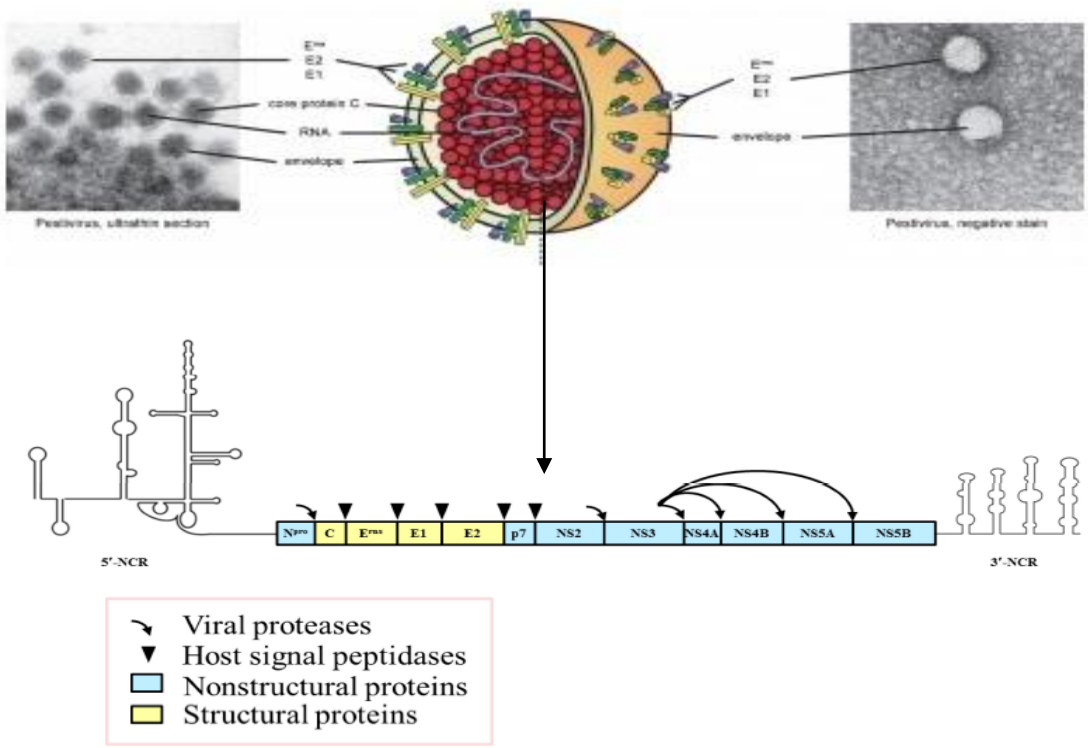


Figure 2. 1: Organization of genome and encoding proteins of CSFV(S. Li et al., 2017; Rangelova & Uttenthal, 2013)

The viral genome of about size 12.3 kb contains noncoding regions at both 5' and 3' ends that play role in viral RNA replication and/or in protein translation and a large open reading frame (ORF) encodes a polyprotein which is processed into four structural proteins and eight nonstructural proteins with the help of viral and cellular proteases(S. Li et al., 2017; Rangelova & Uttenthal, 2013).

2.2 Genetic typing of CSFV

The genus *Pestivirus* consists of four genotypes including CSFV. The other Pestiviruses namely bovine viral diarrhea virus types I and II (BVDV I and II) and ovine border disease virus (BDV) occasionally infect pigs but usually do not spread efficiently outside their ruminant hosts. The CSFV forms a distinctive group though it is closely similar to BVDV and BDV. It can be differentiated serologically or on the basis of genetic similarities.

With advancement of the technology it is possible to determine the relatedness of different virus isolates on the basis of determination and comparison of nucleotide sequences of viral genome fragments which is useful for classification, trace patterns of virus spread and in control strategies and is generally considered superior to antigenic methods. For the classification of CSFV into groups and subgroups, three regions of the genome, 5'-NTR, E2 envelope glycoprotein and NS5B polymerase genes have been most commonly used(Paton et al., 2000). The phylogenetic studies were done on the basis of short fragments, namely a 150 nucleotide (nt) fragment of 5'-NTR, a 190 nt fragment of the E2 encoding region and a 409 nt fragment of the NS5B encoding gene. But now a days sequencing of longer fragments or even full genomes are preferred than that of the traditional fragments as there are affordable sequencing technologies available which is also recommended by the European Union (EU) Reference Laboratory for CSF(Blome et al., 2017). Based on sequence comparison of different genomic regions, CSFVs has been assigned into three main groups (1, 2 and 3) which are sub-divided into ten subgroups. Each of the first and second group has three sub-groups i.e. 1.1, 1.2, 1.3 and 2.1, 2.2, 2.3 respectively. In the case of third group, it has four sub-groups i.e. 3.1, 3.2, 3.3 and 3.4. A database of these sequences is available at OIE Reference Laboratory for CSF, Hanover, Germany. The group 1 comprises the historic isolates from Europe, America and Asia, group 2 includes recent isolates from different regions of Europe and Asia and the group 3 consists of isolates from United Kingdom, Korea, Thailand, Japan and Taiwan(Shivaraj, 2014). Recently a new sub-genotype 1.4 has been added to genotype 1. The newly formed sub-genotype 1.4 is a Cuban isolates which were included in sub-genotype 1.2 before was regrouped on the basis of analysis of full-length E2 sequences as these isolates were found quite divergent from other strains of genotype 1 (Beer, Goller, Staubach, & Blome, 2015).

Table 2. 1: Genogroups of CSFV with the country of their origin:

Genetic Groups	**Sub Groups/ Sub Genotypes**	**Continents/Countries Reported**
1	1.1, 1.2 and 1.3	Historic isolates from Europe, America and Asia.
2	2.1, 2.2 and 2.3	Recent isolates from different regions of Europe and Asia.
3	3.1, 3.2, 3.3 and 3.4	From United Kingdom, Korea, Thailand and Japan/Taiwan respectively.

2.3 Host-virus interaction

During CSFV infection, the envelope glycoproteins E(rns) and E2 are found to be responsible for the binding and entry into the host cell by the pathogen. The cellular receptors heparin sulfate (HS) and laminin receptor (LamR) interact with the viral envelope glycoproteins for attachment of the virus(Chen et al., 2015). The virus entry requires a low-pH environment, depends on dynamin and membrane cholesterol. The Rab proteins Rab 5 and Rab 7 are crucial for the clathrin mediated endocytosis for the entry of the virus(Shi et al., 2016). After the entry, the viral genome is uncoated, released and translated into a polyprotein which is processed by cellular and viral proteases for formation of different viral proteins. In addition the viral genome can also be transcribed into negative strand RNA that will be used as a template for production of progeny positive sense RNA. The virion thus formed are then released from the host cells(S. Li et al., 2017). There is involvement of host cytoskeletons such as actin filaments, microtubules, and intermediate filaments in the process of entry, transport, assembly, and egress of the Flaviviruses(Foo & Chee, 2015). The cellular actin was found to be interacting with the E2 protein hence affecting the early stage of the replication cycle and intracellular transport of CSFV or E2 protein in the cell at the post entry step. A lipid raft-associated scaffold protein i.e. Annexin A2 (Anx2) plays its role in membrane trafficking, aggregation of vesicles, and endosome formation. The CSFV production is promoted by interaction of Anx2 and E2. In addition, the Anx2 interact with NS5A and enhances the assembly of virus. These show the role of Anx2 in multiple steps of life cycle of CSFV. There is also role of host factors in NS5A regulated viral genome synthesis and translation such as, heat shock protein 70 (HSP70) promotes viral RNA replication by interacting with NS5A. Furthermore the eukaryotic elongation factor 1A (eEF1A) interact with NS5A of CSFV resulting in the inhibition of IRES-mediated translation efficiency(S. Li et al., 2017).

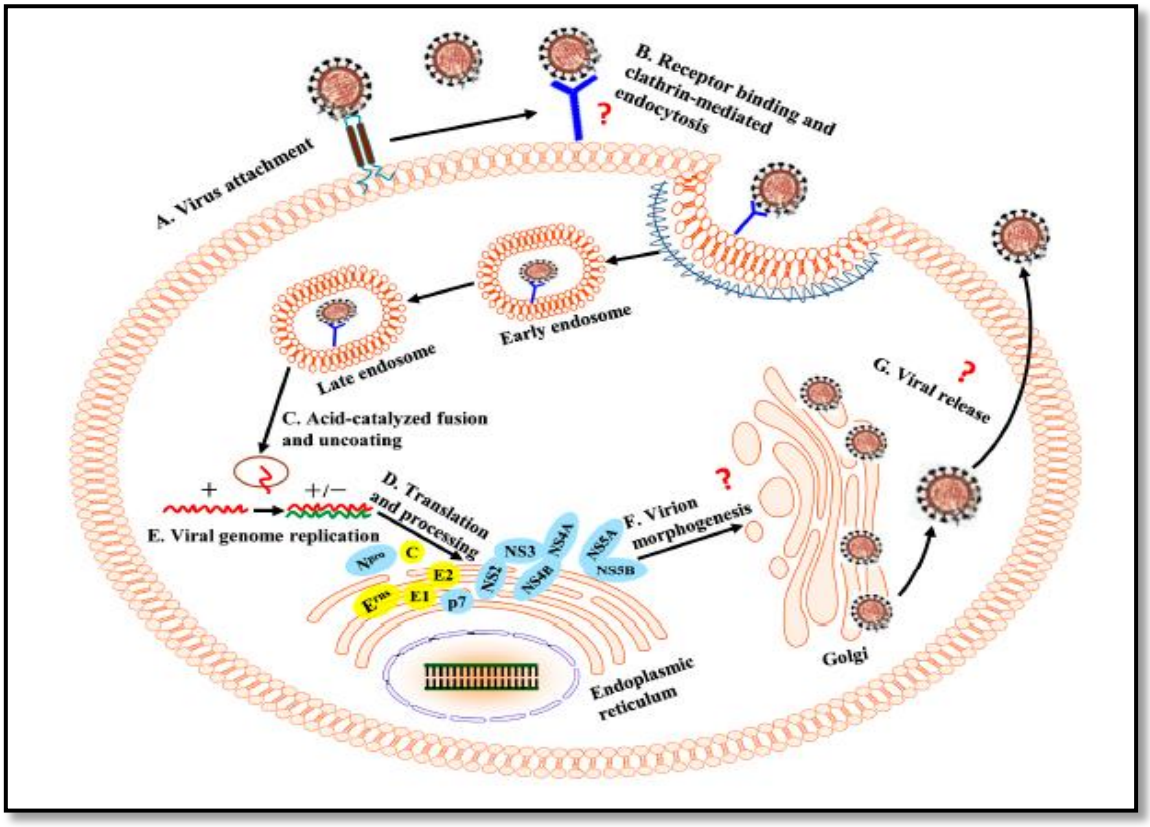


Figure 2.2: Overview of CSFV life cycle

(A) Virus attachment mediated by Interaction between Erns and HS and/or LamR. (B) Clathrin-mediated endocytosis. (C) Fusion of viral envelope and membrane at low pH. (D) Translation and processing of viral proteins. (E) Viral genome replication. (G) Release of mature virions from the cell (source: (Li et al., 2017))

2.4 Effect of viral proteins on host innate immunity

Viruses employ different strategies to evade the host innate immune responses for the successful replication of virus. One of the strategy to escape the type I IFN-induced antiviral mechanism during virus infection is the interaction of N(pro) of CSFV with IFN regulatory factor-3 (IRF-3) or IRF-7 for blocking of type I IFN induction(Fiebach, Guzylack-Piriou, Python, Summerfield, & Ruggli, 2011). The host poly(C) binding protein 1 (PCBP1) was identified as a novel interacting partner of the CSFV N(pro) protein and found to modulate positively to the growth of CSFV by negatively regulating the type I IFN pathway(D. Li, Li, et al., 2013). A protein called hemoglobin subunit beta (HB) was identified as a C protein binding protein which interact and colocalizes with C protein in the cytoplasm. The HB was found to antagonizes CSFV replication via the RIF-I mediated IFN signaling by interacting with the C protein. Whereas, CSFV inhibits expression of HB to block the pathway(D. Li, Dong, et al., 2013). Thioredoxin 2 (Trx2) is a mitochondrion-associated protein that participates in diverse cellular events. The Trx2 was identified as

a novel E2 interacting partner that interact with E2 and inhibits CSFV replication via nuclear factor kappa-light-chain-enchancer of activated B cells (NF-B) signaling, whereas CSFV inhibits protein expresssion of Trx2 to antagonize the antiviral effects(S. Li et al., 2015). A study show that mitogen-activated protein kinase 2 (MEK2), a binding partner of the E2 protein of CSFV promotes CSFV replication through attenuation of the Janus kinase/signal transducers and activators of transcription (JAK-STAT) signaling pathway; a key antiviral pathway involved in innate immunity, by interacting with the E2 protein(Wang et al., 2016). Recently, host guanylate-binding protein 1 (GBP1) was identified as a potent anti-CSFV ISG. It was shown to inhibit CSFV replication depending on its GTPase activity but the CSFV blocks the antiviral activities of GBP1 via inhibition of GBP1 expression(L.-F. Li et al., 2016).

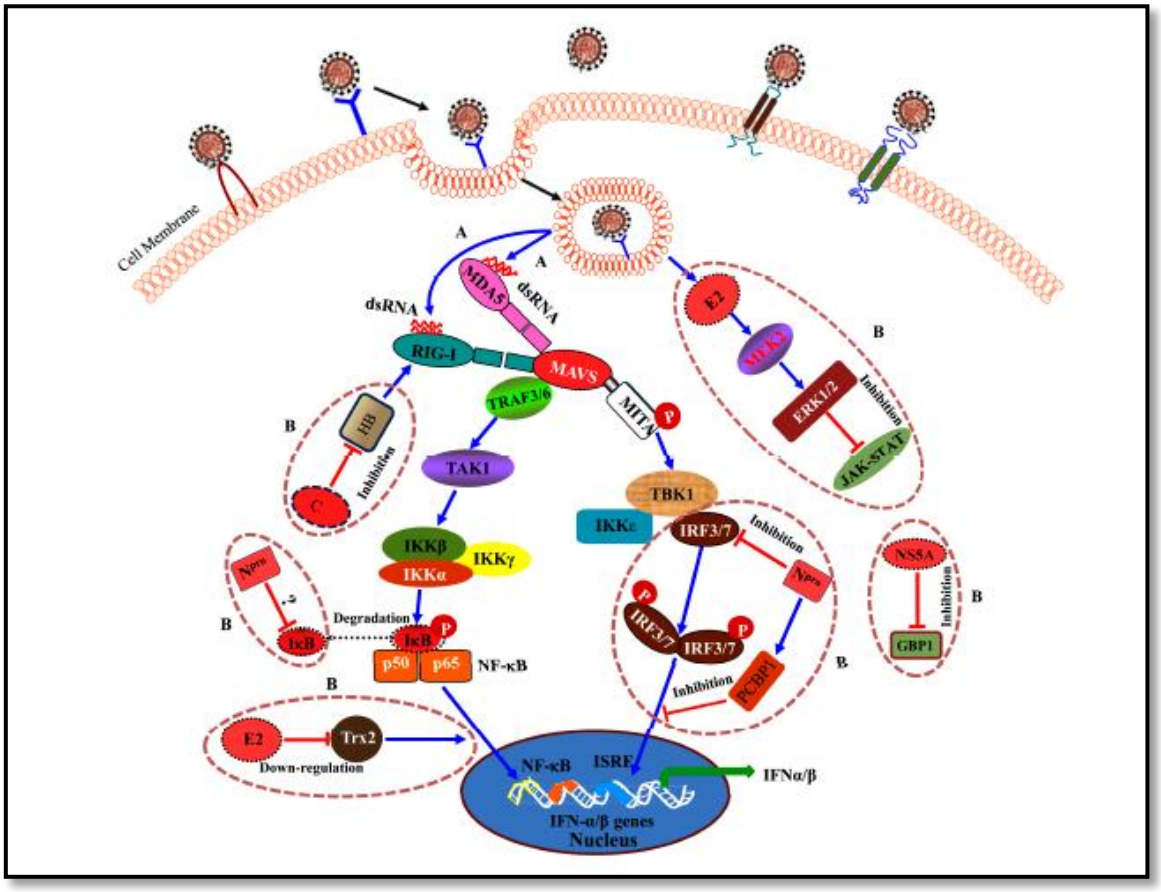


Figure 2.3: Activation and blockage of intracellular signaling pathways of innate immunity during CSFV infection(L.-F. Li et al., 2016)

2.5 Vaccines

A vaccine is a biological preparation that contains an agent that resembles a disease-causing microorganisms which might either be made from weakened or killed forms of the microbe, its toxin or one of its surface proteins. The agent stimulates the body's

immune system to recognize the agent as foreign, destroy it and remember it. After vaccination, the immune system can recognized and destroy more easily any of these microorganisms that it later encounters thereby preventing particular disease. The vaccination works by stimulating the immune system for recognition of invading bacteria and viruses and producing antibodies to destroy or disable them.

Vaccines can be prepared as live or inactivated (killed) products. There are different strategies of vaccine production. Some live vaccines can be prepared by using low virulence, mild, field isolates of a disease causing agent if they have been found safe and effective on administration through unnatural route and result in immunization in host rather causing disease. Other live vaccines can be prepared by modifying the disease causing agents either by passage through laboratory animals, culture media, cell culture, or by using avian embryos to select variant of reduced virulence. Now there is development of recombinant DNA (rDNA) procedures providing some unique opportunities for vaccine production. The virulence related genes of microorganism can be specifically deleted and also we can insert genes coding specific immunizing antigens from a disease causing microorganism into a non virulent vector microorganism for the production of modified live vaccines. There is also development of nucleic acid mediated vaccines which contain plasmid DNA that codes for immunizing antigens from disease causing microorganisms.

Both the live and inactivated vaccines contain antigenic components formulated with adjuvants, stabilizers, antimicrobial preservatives and diluents. The adjuvants used are usually water in oil emulsions, mineral or vegetable oil and emulsifying agent, these enhances the immunizing efficacy of the vaccine. Other adjuvants such as aluminium hydroxide gel of saponin can also be used(OIE, 2014).

Now there are several CSFV vaccines commercially available and have been used successfully to control the CSF disease in many countries worldwide; among them conventional live attenuated vaccines such as lapinised Chinese C-strain or its derivatives and Thiverval strain have been most widely used. The vaccines have shown outstanding efficacy and safety with limitation of not allowing serological differentiation of infected animals from the vaccinated ones; therefore the vaccinated animals are subject to trade restrictions. Now there is development of marker vaccines based on different vector platforms and expression systems to overcome the above limitations(Rossi et al., 2015).

2.5.1 Types of vaccines

2.5.1.1 Live attenuated/modified vaccine (MLV)

The live CSFVs are the first group of commercially available vaccines that have been produced by attenuation of virus through multiple passages in cell culture or in rabbit (lapinised). The most widely used vaccine of this type is “Chinese (C)-strain”. There are also other vaccines of this type available such as; ATCC 131-VR (Argentina), TVM-1 (Czech Republic), GPE (Japan, Singapore), RP 93 (Romania), Thiverval (France) and PAV (Mexico)(Matthias Kramera et al., 2009). The origin of Chinese strain of CSF is exactly unknown. There appears to be various Chinese strains used in vaccine production most of which have been serially passaged hundreds of times in rabbits. It is different from a virulent or its parental strain as there is presence of U-rich insertion of 13 continuous nucleotides in the 3-non coding region; but it is not clear whether this insertion is involved in rendering the C-strain avirulent. Whereas the GPE vaccine strain is developed by serial passage of virulent ALD strain serially through different cell cultures at 30◦C(Van Oirschot, 2003).

2.5.1.2 E2 subunit marker vaccines/DIVA vaccine

This is a new generation of vaccine developed to address the problem of the MLVs in regards to differentiation between infected and vaccination animals (DIVA-principle). It is based on the strategy that the purified form of E2-glycoprotein of CSFV which act as an antigen is capable of inducing a protective immunity in the host. The E2 glycoprotein is produced in cultures of insect cells infected with the baculovirus vector(Matthias Kramera et al., 2009). By using this vaccine, there can be discriminate the infected pigs from the vaccinated one by performing an ELISA that detects antibodies against the Erns protein(Van Oirschot, 2003).

2.6 Diagnosis of Classical swine fever virus:

Diagnosis of disease is very important for the treatment and implementation of control measures for any disease. There are different strategies that can be applied for the diagnosis of classical swine fever. The diagnosis of the disease can be done from clinical sign and symptoms, pathological findings, immunological techniques, virus isolation and molecular techniques.

2.6.1 Clinical sign and symptoms and gross pathological findings

In the field the sign and symptoms become evident for the disease. The predominant signs and symptoms of CSF in all age groups are Pyrexia, huddling of sick animal, dullness, loss of appetite, weakness and conjunctivitis. The disease animals may also

display a staggering gait, ataxia or convulsion(OIE, 2014). Commonly the incubation period of the disease is 7±10 days. The petechial haemorrhages or a purple discoloration of outer skin and mucosaeofthe ears, abdomen and inner thighs are the most typical sign although they are not seen consistently. Disorders of central nervous system and constipation followed by diarrhoea may also be characteristic clinical findings. The severity of clinical signs of the disease largely depends on the age of the host animal and virulence of the infective virus. Usually the young ones are affected more severely than the older ones(Volker Moennig, 2000).

On post mortem of the diseased animal the pathological changes observed most often in lymph nodes, spleen and kidneys. In diseased animal the lymph nodes become swollen and haemorrhagic. Haemorrhages in kidney, urinary bladder, larynx, epiglottis and heart can be observed. CSF virus causes Severe leukopenia and immunosuppression due to classical swine fever virus often leads to secondary enteric or respiratory infections in infected animal(V Moennig et al., 2003).

The clinical and pathological signs of CSF are rather unspecific and confusing with signs and symptoms of other viral diseases such as African swine fever, porcine dermatitis and nephropathy syndrome (PDNS), and post-weaning multisystemic wasting syndrome (PMWS), thrombocytopenic purpura. The sign and symptoms of acute CSF are similar with some bacterial diseases like salmonellosis (especially caused by Salmonella choleraesuis), erysipelas, pasteurellosis, actinobacillosis (caused by Actinobacillussuis) and Haemophilusparasuis hence causes difficulty in distinguishing CSF from the concurrent septicaemic condition. (OIE, 2014).

In spite of the unspecific clinical signs and symptoms of CSF, more than two-thirds of the infected herds were identified on the basis of the clinical signs and symptoms on analysis of 270 outbreaks that occurred in Germany between 1990 and 1998(Fritzemeier et al., 2000).

2.6.2 Classical Swine Fever Virus isolation

As the variability of the clinical signs could not provide firm evidence for unambiguous diagnosis, it must be confirmed by laboratory investigations. Virus isolation is another way for diagnosis of CSF. This technique is more sensitive for detection of the CSFV but slower one. Though there are new advanced methods for the direct detection of CSFV, isolation of virus in cell culture is the gold standard. The virus is best isolated in rapidly dividing porcine kidney (PK-15) cells that seeded on to coverslips along with 2%

suspension of the tonsil in the growth medium. Other pig cell lines whose sensitivity is similar to that of PK-15 and cells free of *P*estiviruses and *Pestivirus* antibodies may be used. Tonsil, spleen, kidney, ileum or lymph nodes of pigs can also be used for isolation of the CSFV among them tonsil is the most suitable one. The virus does not cause a cytopathic effect in the culture cells hence is carried out by an immunostaining method in which the cultures are examined for fluorescent foci by FAT after 24-72 hours or by immunoperoxidase staining after 3–4 days of incubation(OIE, 2014).

2.6.3 Immunological responses

2.6.3.1 Fluorescent antibody test

The fluorescent antibody test (FAT) is a rapid test used for the detection of CSFV antigen in tissue samples such as tonsils, lymph nodes, spleen and kidney. An evaluation of FAT during the 1997/98 outbreak in the Netherlands, showed that the diagnostic sensitivity of the FAT in tissues was found to be approximately 75%, which was compared with a confirmation tests, the immuneperoxidase test (IPT) and virus isolation (VI) test (Bouma et al., 2001). Though the test is relatively easy to perform, an experience staff is required as interpretation of staining is not fully objective. Furthermore to cut the cryosections, a cryostat is needed.

2.6.3.2 Antigen ELISA

This technique has been developed for rapid diagnosis of CSF in live pigs. It is based on the detection of viral proteins by binding them to antibodies in an ELISA plate. This test has low sensitivity and specificity comparing with most of the other diagnostic tests such as virus isolation and RT-PCR. However this technique is relatively simple to perform without need of tissue culture facilities and the result can be obtain within half a day.

The sandwich ELISA and direct FAT were conducted for detection of viral antigen in postmortem tissue samples of affected pigs comprising of spleen (4), tonsil (3) and kidney (3) in outbreak of CSF in Mizoram state of India in 2005/2006. Among the total of ten tissue samples tested, all the samples were found positive for CSFV both by ELISA and the FAT(Barman et al., 2010).

A workshop was held under the Commission of the European Union (EU) at the Community Reference Laboratory, Institute of Virology, Hanover Veterinary School in 1995 for evaluating various ELISA techniques to detect the CSF viral antigen in blood using double antibody sandwich methods and different types of antibodies i.e.

monoclonal and/or polyclonal antibodies. The specificity of the ELISA was found to be good comparing to virus isolation as in only one sample a false positive result was obtained which was found negative by virus isolation. Furthermore, Some false-negative results were seen with the samples collected at up to eight days after inoculation, but all tests for the samples collected between nine and fourteen days post-inoculation were found to be positive(Depner et al., 1995).

2.6.3.3 Antibody detection

Serology has been routinely used for diagnosis of CSF whenever there is suspection of the disease. In CSFV infected pigs, the antibodies are usually detectable from one to three weeks after infection and persist in surviving animal for lifetime. Virus neutralization test (VNT) and enzyme linked immunosorbent assay (ELISA) are the most commonly used tests for the detection of antibody.

The VNT is gold standard technique for antibody detection. It is very useful for discrimination of infection with CSFV or other closely related pestiviruses. However this technique is time consuming and work intensive as there is need of cell culture.

There have been developed several ELISA techniques for the detection of antibodies against the viral E2 glycoprotein using specific monoclonal antibodies, mainly competitive or blocking ELISA and non-competitive ELISA based on monoclonal antibodies. These techniques are widely used for the detection of antibodies during and after the infection of the virus. These techniques have been used for monitoring of CSFV infection and to check the immunization status of pigs and wild boars after vaccination. In general, the ELISA techniques have higher sensitivity but lower specificity as they cross react with antibodies induced by other closely related pestiviruses. The ELISA technique for antibody detection have been extremely used for the diagnosis and monitoring of CSF though it has some limitations as they yield quick result and have large throughput of samples(Greiser-Wilke, Blome, & Moennig, 2007).

2.6.4 Molecular biology of CSF

The CSFV is closely related to the bovine viral diarrhoea virus (BVDV) and border disease virus (BDV) both antigenically and structurally. Hence the polyclonal antibody based diagnosis may fail to assure presence of CSFV as the antibodies against BVDV and BDV may cross-react with CSFV. Therefore the molecular methods that use the primers for amplification of conserved areas of CSFV genes are the best choice for detection and characterization of virus isolates (Barman et al., 2010). A number of PCR assays have been developed that use the reverse transcription-polymerase chain reaction (RT-PCR)

and classical RT-PCR has been proved to be a sensitive and specific tool for diagnosis of CSF(B Hoffmann, Beer, Schelp, Schirrmeier, & Depner, 2005).

2.6.4.1 Reverse-transcription polymerase chain reaction (RT-PCR)

There have been described or are being developed many methods for RT-PCR techniques for the detection of CSFV either early during the incubation period or for a longer period of time in cases where the pigs recover. It is based on the viral nucleic acid detection so can give positive results in cases where virus isolation or other techniques have yielded negative results indicating it is more sensitive technique than others such as: antigen-capture ELISA and FAT. Furthermore, it has become a suitable technique and accepted by numerous nations and the European Union (EU) for screening and confirmation of suspected cases of disease based on its speed and sensitivity. Whereas the RNA extraction is a critical step in RT-PCR analysis as it is difficult to maintain RNA integrity prior to and after extraction. Thus, there might be requirement of treatment of samples prior to RNA extraction and the isolated RNA should be stored carefully for better results. Now there are different methods and RNA extraction kits commercially available(OIE, 2014).

There were different protocols published for the detection of CSFV with the help of RT-PCR in the early nineties; this technique has now become a standard one for routine diagnosis of CSF. A rapid method was developed for the detection of hog cholera virus from infected tissues by using RT-PCR technology in 1991. The sensitivity of the method was found to be of 10(p4) TCID50 of hog cholera virus which was increased with approximately 100-fold on reamplifying with a set of nested primers. On sequence analysis of the PCR product, the tested field isolate was found to be of HCV Alfort strain(Liu et al., 1991). In the same year a diagnostic tool for Pestiviruses was also developed.

Molecular identification and characterization of classical swine fever virus isolate was done in 2012 in Nepal for the first time. Two isolates from outbreak occurred in Makwanpur and Bhaktapur during April 2011 and September 2011 respectively were analyzed using RT-PCR for amplification of full length of E2 gene segment. On sequencing and phylogentic analysis of the amplified gene product, the isolates were found bologning to subgroup 2.2 with highest genetic similarity to isolates from India (Postel et al., 2013). In 2015, RT-PCR was used for the molecular characterization of CSF challenge virus in India. Three regions of CSFV: 5’ NTR, E2 and NS5B were amplified by using the RT-PCR and was obtained required band lengths of 284, 308 and 449bp

respectively. The challenge CSFV was found to be of Group 1.2 on further sequencing and phylogenetic analysis (Kumar, Upmanyu, & Dhar, 2015).

For molecular characterization of lapinized classical swine fever vaccine strain, RT-PCR was employed by Gupta et al. The whole genome of Indian lapinized CSFV obtained from the Division of Biological Products was amplified into nine overlapping fragments using RT-PCR. On further complete sequence alignment and phylogenetic analysis, the strain was found to be 92.6-98.6 % identical to that of the other reported CSFV strains of subgroup 1.1 (Gupta et al., 2011). On molecular characterization of CSFV isolates from Kanrup district of Assam in India during a couple of years from 2012-2014 by using RT-PCR technology, majority of the isolates were found to be of subgroup 2.2 along with 2.1 strains in the northeast part of India. In the study, E2, 5'NTR and NS5B gene segments were amplified and sequenced for characterization of the isolates(Khatoon et al., 2017). Currently the approach of amplification of the targeted gene segment using RT-PCR followed by sequencing and phylogenetic analysis has most widely been used for the characterization of CSFV isolates.

2.6.4.2 Reverse transcriptase nested polymerase chain reaction (RT-nPCR)

For the detection of CSFV in infected pigs RT-nPCR techniques also have been developed. This technique is being used because of its higher sensitivity than that of the primary RT-PCR technique. In this technique two different sets of primers are used in two rounds of PCR for the amplification of single targeted gene. In the first round of primary PCR, larger segment of the gene is amplified which is then used as template for the second round of PCR with the another set of primers which located within the amplified region of first round. Thus the specificity of nPCR is enhanced as there is elimination of false amplification product.

Though the BVDV and BDV cause infections in ruminant which is non-notifiable, but they have capacity to infect pigs which may sometimes cause misinterpretation in diagnostic tests as they are antigenically and structurally close to CSFV. Therefore there is importance or RT-nPCR for the rapid and effective detection and differentiation of Pestiviruses. The work done on comparison of different techniques for the detection of CSFV also shows the nPCR as the most sensitive one. The sensitivity of the n-PCR for detection of CSFV was found 50 times higher than that of the cell culture assay(Sandvik, Paton, & Lowings, 1997). This technique was also found to be more sensitive than the primary conventional PCR; the sensitivity was increased by approximately 100 fold when the primary PCR product was reamplified with a set of nested PCR(Liu et al., 1991).

The RT-nPCR can also differentiate the wild type and attenuated lapinized vaccine strains which can be crucially used in the case of outbreak of CSF in the vaccinated herd of pig farm. This was based on the presence of T-rich insertions sites which exist uniquely in the 3' non translated region of the vaccine strains which are absent in the genome of wild type CSFV. The primers were designed to contain the sequences of the T-rich insertions to distinguish the vaccine strains from the wild one. Though the differential detection can be achieved with the conventional primary PCR, the nested PCR can do it with 10 -100 times more sensitivity than the primary one(Pan et al., 2008). The RT-nPCR technique is the best tool also for the early detection of the CSFV infection so early diagnosis of individual pigs can be done. This technique will be very useful in the trade of pigs as they can be confirmed free of CSF disease with the help of this technique. In a study, an infected pig was found positive 2.8 days (average) earlier with RT-nPCR than the technique of virus isolation in whole blood. The virus was still detected by using the RT-nPCR 2 days (average) after the end of the positive virus isolation in the blood. This indicates that the positive period of the RT-nPCR is longer than the positive period of the virus isolation in the blood(Dewulf et al., 2004).

2.6.4.3 Real time RT-PCR

A sensitive, specific, fast and reliable strategy is very important for an effective control of CSF outbreaks. For this, the RT-qPCR is the most suitable diagnostic tool can be used. Though the classical RT-PCR technique has proved to be a sensitive and specific diagnostic tool, it bears risk of cross contamination as it includes gel-based systems for the detection of amplified PCR products. There is no need of gel based systems in RT-qPCR. It detects the accumulation of amplicon during the exponential phase of the PCR reaction whereas in the classical RT-PCR, only the end- point of the PCR reaction is evaluated. In RT-qPCR, fluorogenic mechanism is employed for the detection of amplification process. There are two types of fluorogenic mechanisms used in detection of CSFV. PCR can be evaluated in real time either by using a fluorescent intercalating dye, such as SYBR green or with TaqMan approach. The SYBR green confirms amplification of target gene segment by fluorescing brightly when it bound to the double stranded target DNA but it does not bind to single stranded DNA. The specificity of the reaction can be determined with the help of the melting point of the product which can be determined at the end of the amplification process. Differing in the melting point indicates the presence of contaminating products due to contamination, mispriming, primer dimers and others. Indirect monitoring of the RT-qPCR uses the TaqMan probes. The probes are the oligonucleotides which contain a fluorescent dye

(which act as reporter) on 5' base and a quenching dye on the 3' base. When these two are in close proximity, there is no emission of any fluorescence. The TaqMan probes are designed to anneal to an internal region of a PCR product so it can be cleaved by the 5' exonuclease activity of DNA polymerase during replication of the template on which the probe is bound as a result there is emission of fluorescence. The fluorescence emission increases with increase in number of cycles(Greiser-Wilke et al., 2007).

SYBR Green I Dye

SYBR Green I Dye is the most commonly used dye for real time PCR which bind to double stranded DNA (dsDNA). When the dye is free in solution, it exhibits minimal fluorescence, whereas on binding with dsDNA the fluorescence increases up to 1000 fold. During amplification process the fluorescent signal increases in proportion to the amount of dsDNA generated. The SYBR Green is compatible for use with any real time cycler as its excitation and emission maxima are at 494 nm and 521 nm respectively(Bio-Rad).

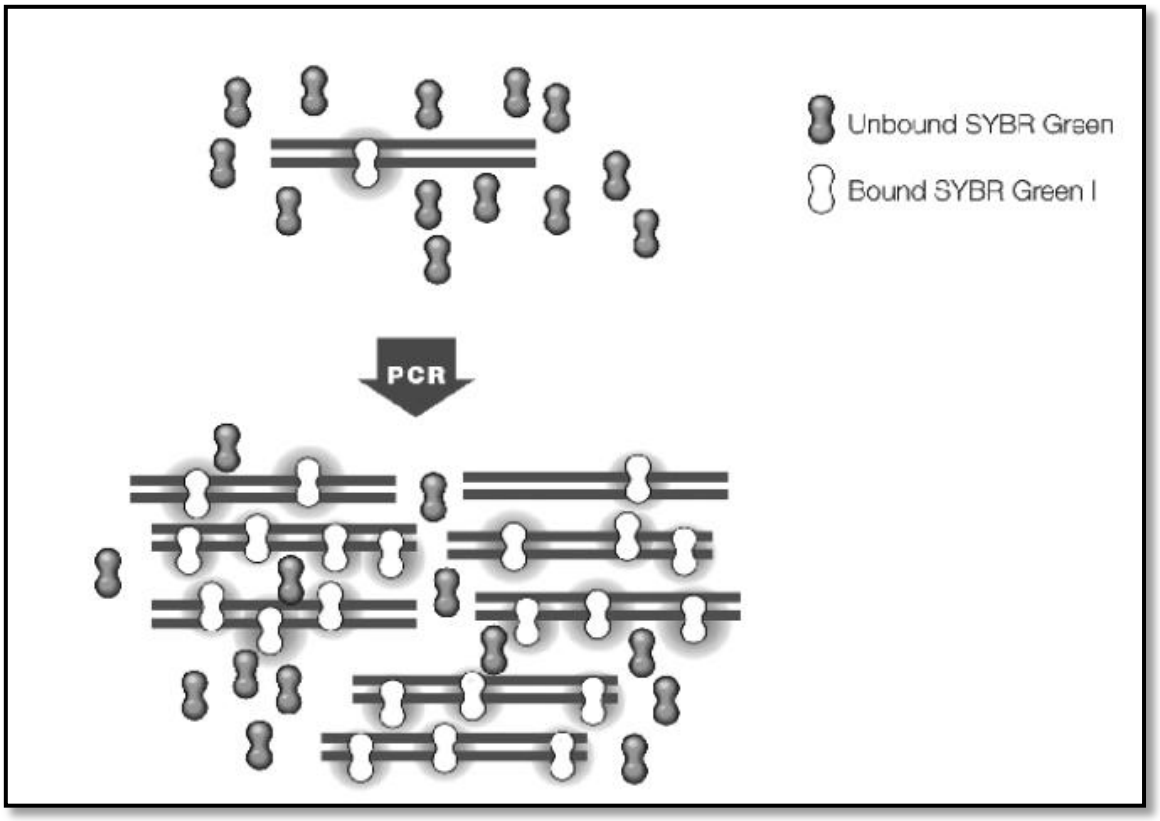


Figure 2.4: Binding of SYBR with dsDNA during real time PCR(Bio-Rad)

qPCR analysis

There are various methods for interpreting Real Time PCR data; among them relative quantification and absolute quantification have been most commonly used. These methods used CT values obtained during qPCR. In absolute quantification technique, the CT values of test samples are used to compare with that of the standards of known quantity. A standard curve is used for comparing the CT values which gives the copy number or concentration of the test samples. Whereas in the case of relative quantification, the ratio of the relative amount of (fold difference) of a target nucleic acid in test vs. control samples can be determined with comparison of CT values of test samples to those of control samples. The absolute quantification is used for pathogen detection or copy number analysis whereas the relative quantification is commonly used for gene expression studies.

In real time PCR, melt-curve analysis is done for identification of reaction products i.e whether the products are specific or nonspecific one. This analysis is valuable in qPCR as the presence of nonspecific products and primer dimers severely reduce the amplification efficiency and accuracy of data. The melt peak obtained after completion of the qPCR can confirm the specificity of the chosen primers. It also discriminate the desired amplified product from primer dimers and nonspecific products as the primer dimers are smaller in size therefore melt lower temperature than that of the desired product whereas the nonspecific amplification occurs with melting temperature above or below of that of the desired product(Bio-Rad).

There are different protocols of real time reverse transcription polymerase chain reaction (rRT-PCR) available which have been used in routine diagnosis of CSF. A fully validated, ready to use assay of rRT-PCR for simple and rapid detection of CSFV was developed by Hoffman et al ; which was multiplexed for simultaneous detection of an internal control(B Hoffmann et al., 2005). There was also a multiplex rRT-PCR technique developed for quantitative and differential detection of wild type viruses from the C-strain vaccine with the sensitivity of 41.8 and 81.5 copies/mL viral RNA respectively(Zhao et al., 2008).

A study was conducted for analysis of results of RT-qPCR ring trial from 10 European laboratories routinely involved in CSF diagnosis for the detection of CSFV genomic RNA. The results from all the participants laboratories were obtained within the acceptable range. In general, all participants produced results within the acceptable range. The tested FLI assay, several in-house assays, and the commercial kits were found to be

highly sensitive and specific. Nevertheless, some in-house systems had unspecific reactions or suboptimal sensitivity with only a single CSFV genotype(Bernd Hoffmann et al., 2011). The RT-qPCR was found to be efficient for detection of CSFV in different tissue samples such as blood, tonsils, and kidney(Dias et al., 2014).

There was a RT-qPCR assay developed which is suitable for a double check strategy for the detection of CSFV in outbreak of CSF. In contrast to others, the target for this assay was a polyprotein –encoding genome region, NS5A gene. The assay was multiplexed with a β-actin detection system as an internal control. This technique was found to be reliable in detection of CSFV genome independent of the 5'NTR gene segment(Leifer, Blome, Beer, & Hoffmann, 2011). There was also development of an assay based on strategy of SYBR Green coupled to melting curve analysis for the detection of CSFV. For validation of this assay, the analytical and diagnostic performances of two real-time PCR instruments were compared. The assay was found to be highly specific for major genotypes of CSFV and it was also considered as a highly sensitive as it detected the CSFV in tissue homogenate and serum samples from naturally and experimentally infected animals whereas the CSFV was not detected in samples from non infected animals. The primers specific for NS5B protein were used for the detection of virus (IgnacioNuñezbLlilianneGangesb, 2011).

Chapter III

MATERIALS AND METHODS

3.1 Sample collection:

For this research work, classical swine fever vaccine samples available in Nepal were collected. We found two types of lapinized live attenuated vaccines; one developed by Hester Biosciences Nepal Private Limited, Nala Ugrachandi and another one by Central Biological Production Laboratory, Tripureshwor, Kathmandu. Total of four samples were collected; three were provided by Hester Biosciences Nepal Private Limited) and One from market that was developed by the Central Biological Production Laboratory. A positive control (a vile containing a seed virus used for vaccine development by Hester) was also provided by Hester Biosciences Nepal Private Limited). The three samples provided by Hester Biosciences Nepal Private Limited samples were of same batch and named as Hester Vaccine 1 (HV1), Hester Vaccine 2 (HV2) and Hester Vaccine 3 (HV3). Whereas, the vaccine sample from market was labeled as MV. The seed virus that Hester had used for production of vaccine was used as positive control and named as PC.

3.2 Sample transport to lab of the department

The vaccine samples collected were transported to the lab of Central Department of Biotechnology, Kirtipur, Kathmandu with ice pack and were stored in refrigerator at -80◦C.

3.3 RNA extraction

For extraction of viral RNA, the samples were taken out from -80°C refrigerator and were thawed. Then the RNA of the virus was extracted using GeneJET RNA Purification Kit (Thermo Scientific, Cat. No. K0731) according to manufacturer's specification. For viral RNA extraction, the working station and equipment (vortex, centrifuge etc.) was wiped with 70% ethanol. The thawed sample was then resuspended with 500uL nuclease free water with vortexing. Then 200uL of the resuspended sample was mixed with 600uL of lysis buffer in a sterile eppendorf tube, vortexed for 1 minute and was centrifuged for 7 minutes at 12300 rcf. After the centrifugation, the supernatant was transferred into a new RNase-free microcentrifugetube. Then 300uL absolute ethanol was added to the supernatant and was mixed by pipetting. 6oouL of the lysate was transferred to GeneJET RNA Purification Column inserted in a collection tube and the

column was centrifuged at 12000rcf for 1 minute. The flow-through was discarded and the purification column was placed back into the collection tube. The remaining lysate was also processed in the same manner. The purification column was placed into a new collection tube, 700uL of wash buffer 1 was added to the column, centrifuged for 1 minute at 12000rcf, discarded the flow-through and placed the column back into the collection tube. 6oouL of wash buffer 2 was added to the column, centrifuged for same time and speed, discarded the flow-through and placed the column back into the collection tube. Again 250uL of wash buffer 2 was added to the column, centrifuged for 2 minutes at 2000rcf and discarded the flow-through and the column was placed into a new sterile RNase-free microcentrifuge tube. Finally, 50uL of nuclease free water was added to the center of the purification column membrane and centrifuged at 12000rcf for 1 minute to elute RNA. Again, the column was placed into a new microcentrifuge tube and RNA was eluted with another 50uL of nuclease free water. Then the collected RAN was stored at -80oC and used for further application.

3.4 cDNA synthesis

cDNA was synthesized from the viral RNA extracted. SuperScript™ III Reverse Transcriptase (Invitrogen, Cat. No. 18080-044) was used for synthesis of cDNA. For these, two reaction mixtures were prepared. Reaction mixture-1 of volume 10uL was prepared by adding 1uL of 10mM dNTP, 1uL of random hexamer and 8uL of the extracted RNA to a sterile nuclease-free microcentrifuge tube. Then the mixture-1 was heated at 65oC for 5 minutes. Reaction mixture-2 of volume 10uL was prepared by adding 4uL of 5X First-Strand Buffer, 1µl of 0.1 M DTT, 4uL of nuclease free water and 1uL of SuperScript™ III RT (200 units/µL) to another sterile nuclease-free microcentrifuge tube and mixing the contents by pipetting gently up and down. The mixture-1 and 2 was mixed together and incubated the tube at 25°C for 5 minutes for annealing of random hexamers, at 50°C for 50 minutes for synthesis of cDNA and heating at 70°C for 15 minutes for inactivation of the enzyme. Thus synthesized cDNA was stored at -20°C and used for PCR amplification.

Thermal cycling condition for cDNA synthesis

For cDNA synthesis, reverse transcription was carried out in the thermal cycler with the given cyclic conditions

Step I : 65°C for 5 min

Step II : 25°C for 5 min

Step III : 50°C for 50 min

Step IV : 70°C for 15 min

The cDNA thus synthesized was stored at -20°C.

3.5 PCR amplification

For amplification of the targated gene segment, nested PCR was performed. For nested PCR two rounds of PCR was conducted, for which two sets of primers were used for amplification of the targated gene segment. In this research work two gene segments: 5'-Nontranslated gene (5'NTR) and E2 gene segment were amplified for which two sets of primers (Table-3.1) were used for each gene. For each reaction 2X PCR master mix (Thermo Scientific) was used.

Table 3.1: Primers used for amplification of 5'NTR and E2 regions by nested PCR

Gene	PCR round	Primer	Primer sequence (5'----3')	Annealing temperature (Ta)	Nucleotide position
5'NTR	PCR-1	Forward: 5'NTR-F	CTAGCCATGCCCWYAGTAGG	60.5°C	94–113
		Reverse: 5'NTR-R	CAGCTTCARYGTTGATTGT	50.9°C	514–496
	PCR-2	Forward Internal: 5'NTR-FI	AGCTCCCTGGGTGGTCTA	58.4°C	146–163
		Reverse Internal: 5'NTR-RI	TGTTTGCTTGTGTTGTATA	48.7°C	417–399
E2	PCR-1	Forward: E2-F	AGRCCAGACTGGTGGCCNTAYGA	64.7°C	2228–2250
		Reverse: E2-R	TTYACCACTTCTGTTCTCA	50.9°C	2898–2880
	PCR-2	Forward Internal: E2-FI	TCRWCAACCAAYGAGATAGGG	57.4°C	2477–2497
		Reverse Internal: E2-RI	CACAGYCCRAAYCCRAAGTCATC	61.1°C	2748–2726

(Barman et al., 2010)

Primer resuspension

The primers ordered were received in a lyophilized state. First master stock of 100X (100uM) was prepared by resuspending each primers in 250uL of nuclease free water as indicated by manufacturer (Macrogen). The resupended mixture was left for 1 hour at room temperature. Then the master stock was diluted by adding 90uL nuclease free water to 10uL of the master stock forming working stock of 10X (10uM). Thus formed working stock primer solution was used for PCR reaction.

3.5.1 First round PCR (PCR-1)

For first round of PCR first sets of primers were used for each gene amplification. For 5'NTR gene amplification, forward primer 5'NTR-F and reverse primer 5'NTR-R were used. For amplification of the E2 gene segment, forward primer E2-F and reverse primer E2-R were used. Total of 30uL PCR reaction mixture (Table-3.2) was prepared for each gene amplification with different PCR condition (Table-3.3). The negative control consisted of nuclease free water instead of cDNA template. Then the PCR tubes containing the reaction mixture were tapped gently, short spun and PCR was carried out by transferring them to the thermal cycler.

Table 3. 2: Reaction mixture of PCR-1 for 5'NTR and E2 gene segments

5'NTR gene		**E2 gene**	
Reagents	**Volume (1X)**	**Reagents**	**Volume (1X)**
Master mix (2X)	15uL	Master mix (2X)	15uL
5'NTR-F	1.5uL	E2-F	1.5uL
5'NTR-R	1.5uL	E2-R	1.5uL
Template cDNA	3uL	Template cDNA	3uL
Nuclease free water	9uL	Nuclease free water	9uL
Total	**30uL**	**Total**	**30uL**

Table 3.3: PCR condition of PCR-1 for 5'NTR and E2 gene segments

	5'NTR			**E2**		
Step	**Temp.**	**Time**	**Cycles**	**Temp.**	**Time**	**Cycles**
Initial denaturation	95°C	2 min	1	95°C	2 min	1
Denaturation	95°C	30 sec		95°C	30 sec	
Annealing	50°C	45 sec	34	56°C	45 sec	34
Extension	72°C	1 min		72°C	1 min	
Final extension	72°C	1 min	1	72°C	1 min	1

3.5.2 Second round PCR (PCR-2)

For second round PCR second sets i.e. Internal primers were used. The primary PCR amplicon from first round of PCR was used as a template for each gene amplification. Total PCR mixture of volume 30uL (Table-3.4) was prepared for each gene amplification with different PCR conditions (Table-3.5). The PCR was then carried out for specific amplification of the 5'NTR and E2 gene segment at given PCR condition. The PCR products obtained were then stored at 4°C for further processing.

Table 3.4: Reaction mixture of PCR-2 for 5'NTR and E2 gene segments

5'NTR gene		**E2 gene**	
Reagents	Volume (1X)	Reagents	Volume (1X)
Master mix (2X)	15uL	Master mix (2X)	15uL
5'NTR-FI	1.5uL	E2-FI	1.5uL
5'NTR-RI	1.5uL	E2-RI	1.5uL
Primary PCR amplicon	3uL	Primary PCR amplicon	3uL
Nuclease free water	9uL	Nuclease free water	9uL
Total	**30uL**	**Total**	**30uL**

Table 3.5: PCR condition of PCR-2 for 5'NTR and E2 gene segments

	5'NTR			**E2**		
Step	**Temp.**	**Time**	**Cycles**	**Temp.**	**Time**	**Cycles**
Initial denaturation	95°C	2 min	1	95°C	2 min	1
Denaturation	95°C	30 sec		95°C	30 sec	
Annealing	56°C	45 sec	34	58°C	45 sec	34
Extension	72°C	1 min		72°C	1 min	
Final extension	72°C	1 min	1	72°C	1 min	1

3.6 Agarose gel electrophoresis

The amplified PCR products were run on 1.5% agarose gel prepared in 1X TAE (Tris base, acetic acid and EDTA) and stained with ethidium bromide. The 1.5% agarose gel was prepared by dissolving 0.75gm of agarose in 50mL of 1X TAE buffer. Three microliters of the PCR products were mixed with 1uL of loading dye and loaded in gel well. DNA ladder of 100bp (GeneRuler 100 bp DNA Ladder) was used as a reference. The gel was

then run at 80 volts for 1 hour. After completion of gel electrophoresis, the gel was visualized under UV Trans Illuminator and gel doc (MS major science UVDI).

3.7 Sequencing and sequence analysis

The PCR products with required band size were sent to Xcelris Pvt. Ltd., Ahmedabad for bidirectional sequencing. The sequences obtained were viewed using the software Chromas, contig was formed from both forward and reverse sequences using BioEdit, then BLAST tool was used for comparing the tested samples with existing database in National Center for Biotechnology (NCBI).

3.8 Phylogenic tree construction

The phylogenic tree was constructed by aligning sequence with ClustalW algorithm and Test Maximum Likelihood Tree using the software MEGA-X. The sequences available in GenBank from different parts of the world (Table), obtained from blast result were included in the multiple sequence alignment and subsequent construction of the phylogenetic tree. Different phylogenetic trees were constructed for 5'NTR and E2 sequences.

Table 3.6: Reference strains obtained from blast of E2 sequence

Isolate Name	Country	Year of Isolation	GenBank Acc. No.
LPC/AHRI	Taiwan	2004	AY526732.1
LPC	Taiwan	2001	AF352565.1
HeN-AY	China	2009	GQ454793.1
LPC	Taiwan	1995	U35740.1
Barra do Corda	Brazil	2008	KX431227.1
Alfort A19	France	1997	U90951.1
GPE	Japan	1995	D49533.1
Alfort187	-	-	NC_038912.1
CSF0741	Japan	1966	MK026454.1
Riems	Switzerland	-	AY259122.1
Chinese strain	-	-	Z46258.1
KNU-1823-2	South Korea	2017	MK121887.1
TLL-Indo	Indonesia	2013	KX130940.1
CSFV-JY-2010	China	2010	HQ380243.1
Thiveral	France	-	EU490425.1

Table 3. 7: Reference strains obtained from blast of 5'NTR sequence

Isolate Name	Country	Year of Isolation	GenBank Acc. No.
LPC	Taiwan	-	AF352565.1
VN91	Vietnam	1991	LC374604.1
Eystrup	-	-	AF326963.1
Strain 39	China	-	AF407339.1
Rovac	USA	1994	KJ873238.1
Koslov	-	2013	KF977610.1
Shimen/HVRI	China	-	AY775178.2
HCLV	-	-	AF091507.1
Chinese strain	-	-	Z46258.1
LK-VNIUViM	-	-	KM522833.1
Lapinized vaccine strain	India	-	EU857642.1
Thiverval	France	-	EU490425.1
4/7 P-2	India	-	EF051174.1
C/HVRI	China	-	AY805221.1
Alfort A19	-	-	U90951.1
Alfort 187	-	-	NC_038912.1

3.9 SYBR Green real time PCR

The real time RT-PCR was performed by using Maxima SYBR Green/ROX qPCR Master Mix(2X) for the amplification of segment of NS5B gene. On perfrming the 2-step RT-qPCR, first of all cDNA was synthesized from the viral RNA extracted from the samples; the cDNA was then used as template for amplification of the targeted gene segment by real time PCR. The amplification of a positive control and five test samples with 25uL reaction mixture for each sample was done by using Bio-Rad PCR machine. The primers used for amplification of target genes are as follows:

Forward Primer: NS5B-F= CCTGAGGACCAAACACATGTTG, Tm=62.1◦C

Reverse Primer: NS5B-R= TGGTGGAAGTTGGTTGTGTCTG, Tm= 62.1◦C

(Pérez et al., 2011).

The PCR reaction mixture of 25uL (Table-3.8) per well was prepared for amplification of NS5B gene segment of test samples. The PCR plate was then loaded to Real-Time PCR machine (BIORAD-CFX96 Touch™ Real –Time PCR Detection System) and proceed for amplification of NS5B gene of different test samples with the required PCR conditions

(Table-3.9). In negative control equal volume of nuclear free water was used instead of cDNA template.

Table 3. 8: Reaction mixture of real time PCR for amplification of NS5B gene segment

Reagents	**Volume (1X)**
Maxima SYBR Green/ROX qPCR Master Mix (2X)	12.5uL
NS5B-F (10uM)	1uL
NS5B-R (10uM)	1uL
cDNA 5uL	5uL
Nuclease free water	5.5uL
Total	**25uL**

Table 3.9: Real time PCR condition

Step	**Temperature (°C)**	**Time**	**Number of cycles**
Enzyme activation	95	10 min	1
Initial denaturation	95	30 sec	
Annealing	58	30 sec	40
Extension	72	35 sec	
Melt curve	95	5 min	

Chapter IV

RESULTS

4.1 RNA extraction

The RNA was extracted from the positive control and all the vaccine test samples i.e HV1, HV2, HV3 and MV by using GeneJET RNA Purification Kit (Thermo Scientific) according to manufacturer's specification. Then the concentration and quality of the extracted RNA was determined by using nanodrop. The concentration of RNA of tested samples were found between 0.29ng/uL to 24.05ng/uL and that of positive control was found to be 135.15ng/uL (Table-4.1). The optical density (OD) ratio at the wave length 260/280 nm of tested samples were obtained in the range of 0.37 to 2.13 and that of positive control was 2.03 (Table-4.1) indicating the extracted RNA from some samples is pure and some with protein contamination.

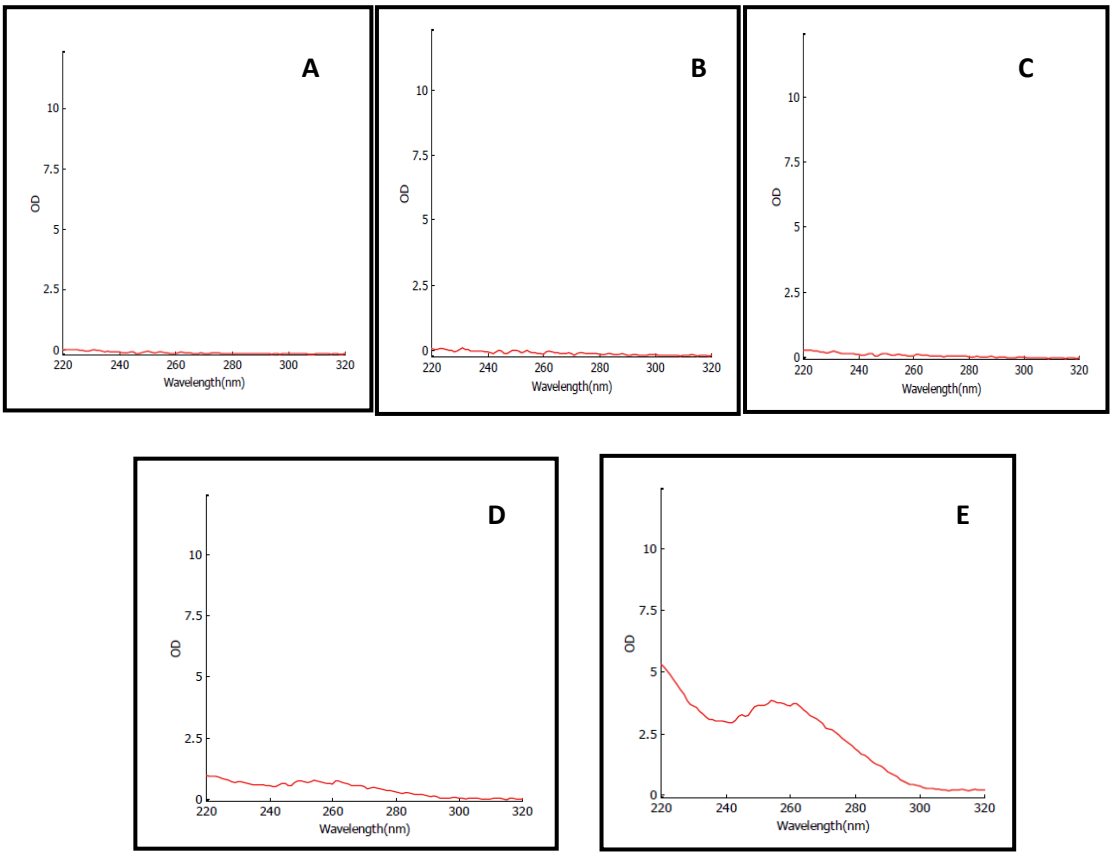


Figure 4.1: Graph showing absorbance at different wavelengths (A) HV1, (B) HV2, (C) HV3, (D) MV, (E) PC

Table 4.1: Concentration and purity of the extracted RNA

S.N.	Sample name	Absorbance		Concn.(ng/uL)	Ratio (260/280)
		260nm	280nm		
1.	HV1	0.154	0.141	0.29	0.37
2.	HV2	0.021	0.013	2.58	1.14
3.	HV3	0.152	0.149	1.75	0.94
4.	MV	0.618	0.298	24.05	2.13
5.	PC	3.596	1.884	135.15	2.03

4.2 Synthesis of cDNA

The cDNA was synthesized by using SuperScript™ III Reverse Transcriptase (Thermo Fisher Scientific) using the extracted RNA as a template. Then after the synthesized cDNA was quantified as well as their purity was checked using nanodrop. The concentration of the cDNA of tested samples was found between 780.72 to 983.07 ng/uL and positive control with 1137.31 (Table-4.2) which is the satisfactory yield. The purity of cDNA was checked by determining the OD ratio of cDNA at wave length 260/280 nm and was found to be pure enough as the ratio was around 1.8 (Table-4.2).

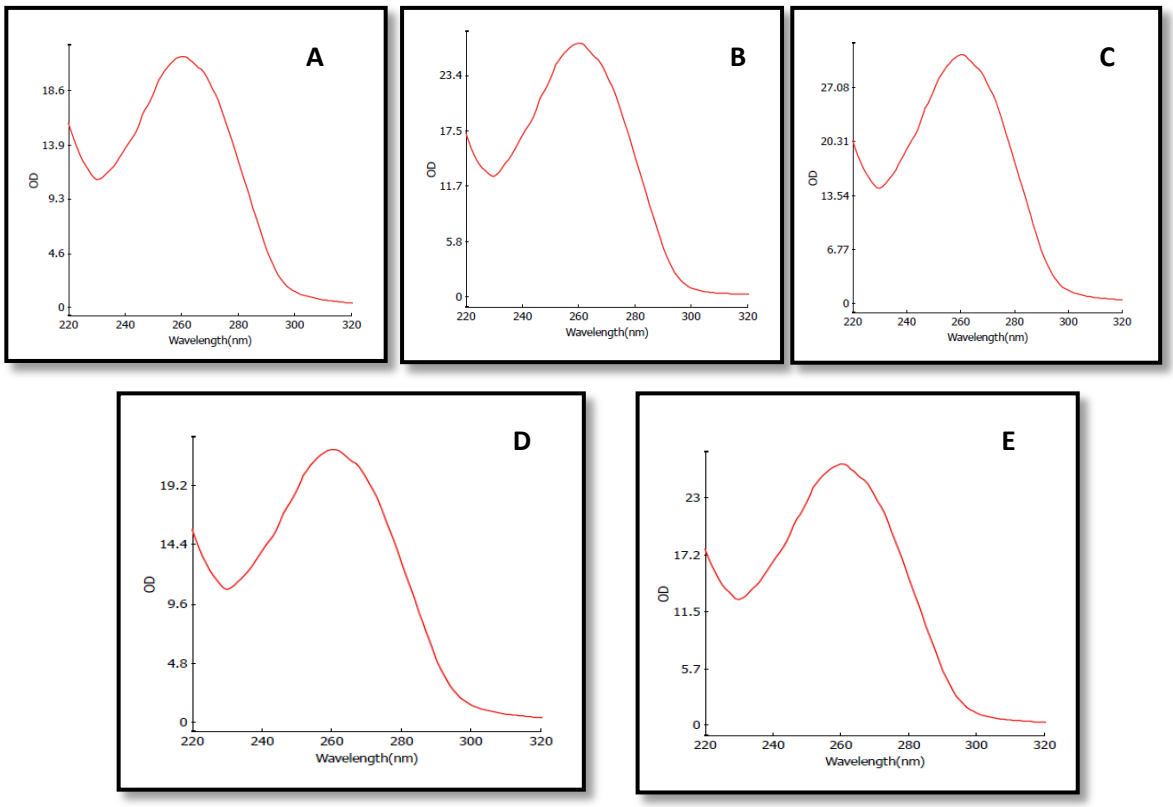


Figure 4. 2: Graph showing maximum absorbance at 260nm(A) HV1, (B) HV2, (C) HV3, (D) MV, (E) PC

Table 4.2: Concentration and purity of cDNA

S.N.	Sample name	Absorbance		Concen. (ng/uL)	Ratio (260/280)
		260nm	280nm		
1.	HV1	21.440	12.533	780.72	1.73
2.	HV2	26.772	14.784	964.50	1.82
3.	HV3	31.120	17.846	804.26	1.76
4.	MV	22.082	12.963	983.07	1.72
5.	PC	26.369	14.916	1137.31	1.78

4.3 Amplification of 5'NTR and E2 regions of CSFV by nested PCR

The cDNA synthesized were subjected for amplification of targeted gene segment of CSFV by using previously published specific primers. There was use of two sets of primers for amplification of a targeted region by nested PCR. In first round of PCR amplification, there was no any band observed on visualizing the 1.5% agarose gel under gel doc. Then second round of PCR was performed by using second sets of primers for amplification of 5'NTR and E2 region. The second round PCR product after agarose gel electrophoresis showed required band size on comparision with 100bp DNA ladder. The band size of 271bp was resolved for positive control and all the test samples for both the gene segments i.e 5'NTR and E2 except for negative control where there was no any band was observed; which indicated that all the tested samples are CSFV positive.

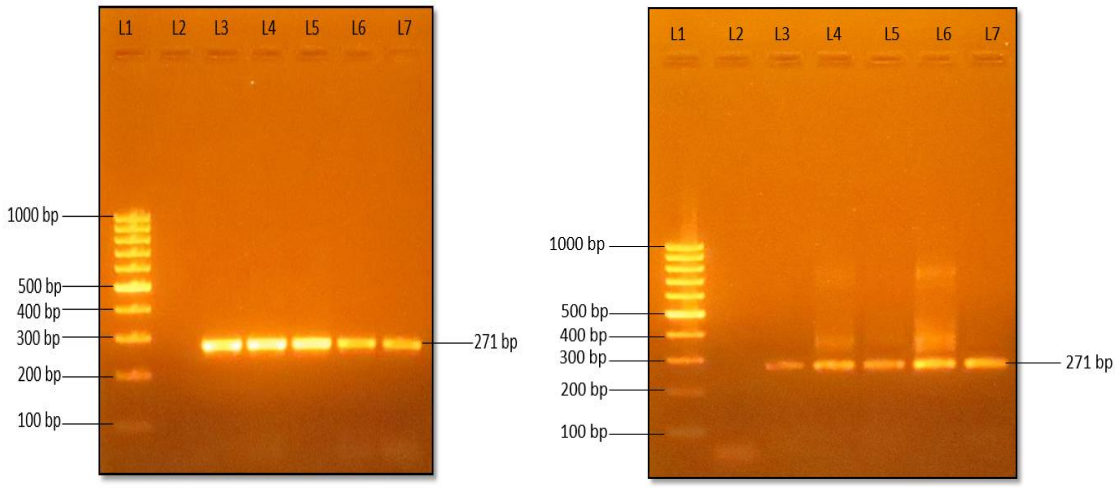
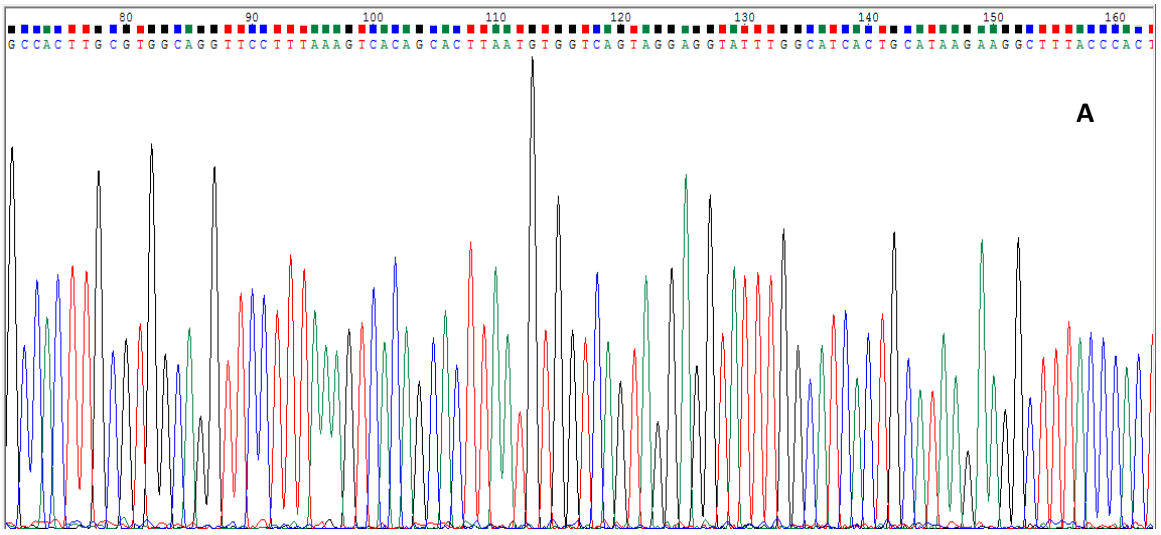
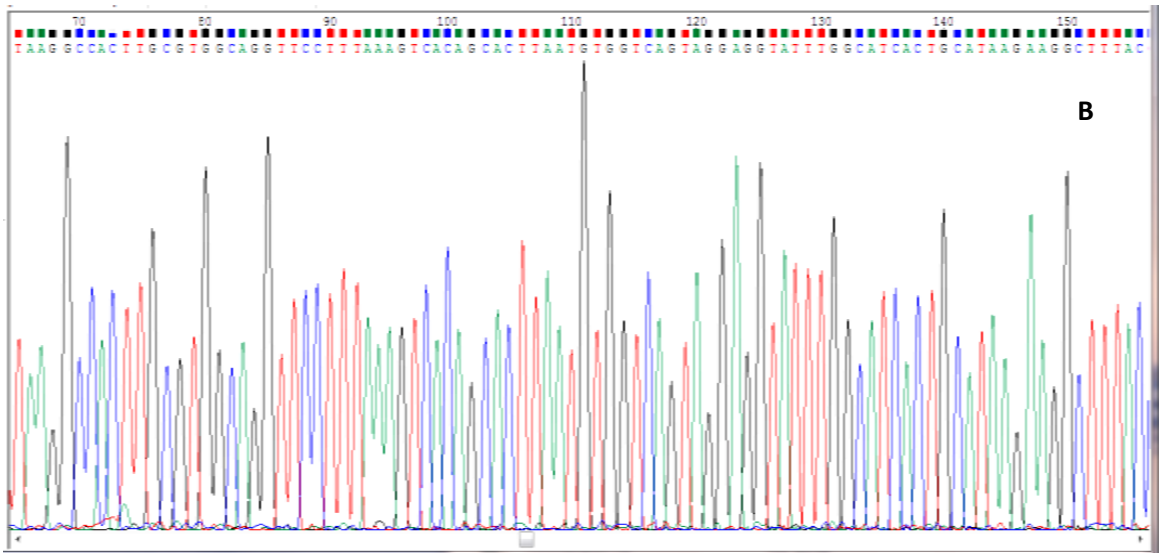


Figure 4. 3: Agarose gel electrophoresis for amplified PCR products(A-for E2gene), (B-for 5'NTR gene) L1- 100 bp ladder, L2- Negative control, L3- Hester Vaccine sample 1, L4 – Hester vaccine sample 2, L5-Hester vaccine sample 3, L6- Market vaccine sample, L7- Positive control

4.4 Sequence analysis

The final PCR products of positive control and all the tested samples that have confirmed CSFV positive by nested PCR were sent to Pvt. Ltd., Ahmedabad for bidirectional sequencing. We received 20 chromatograms from bidirectional sequencing of 5'NTR and E2 gene segments of 5 samples. The chromatograms were viewed by using chromas software and found as follows:





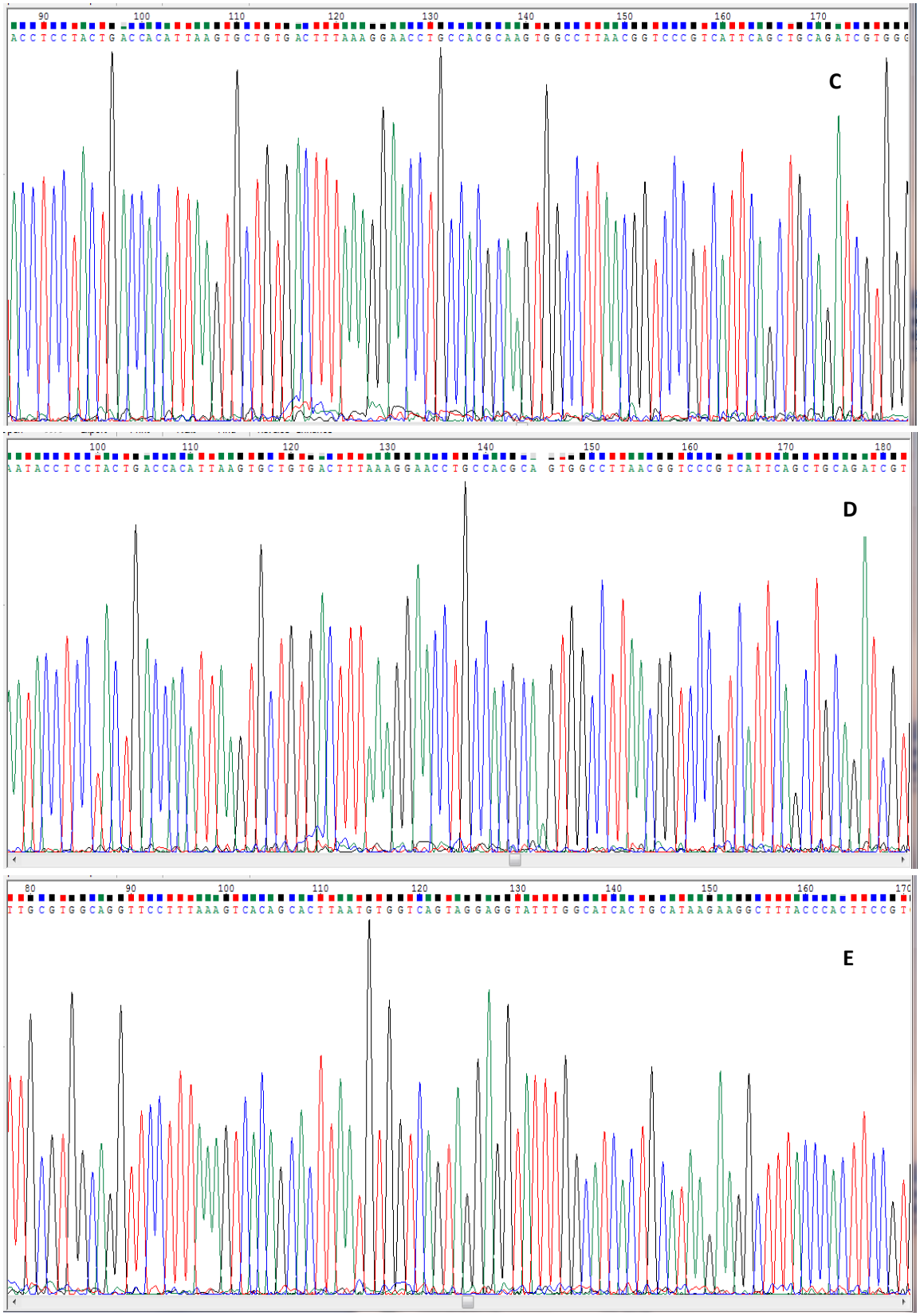
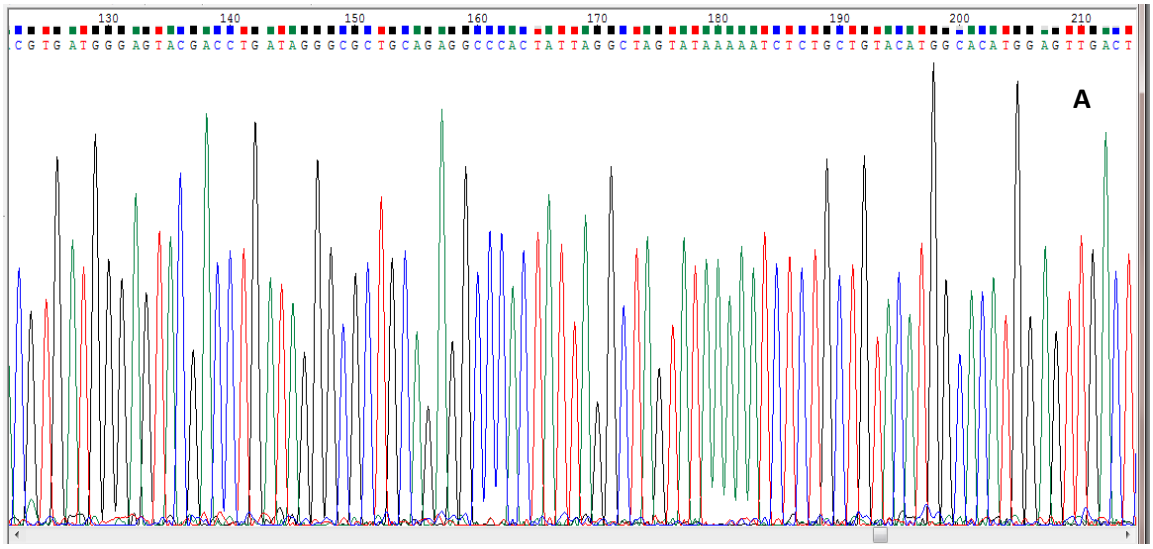
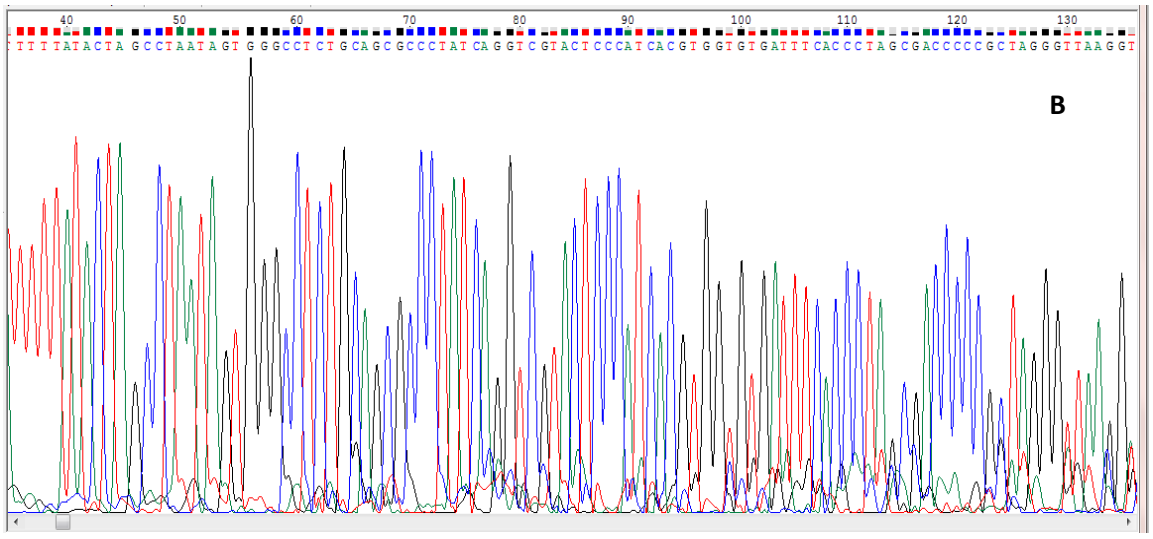
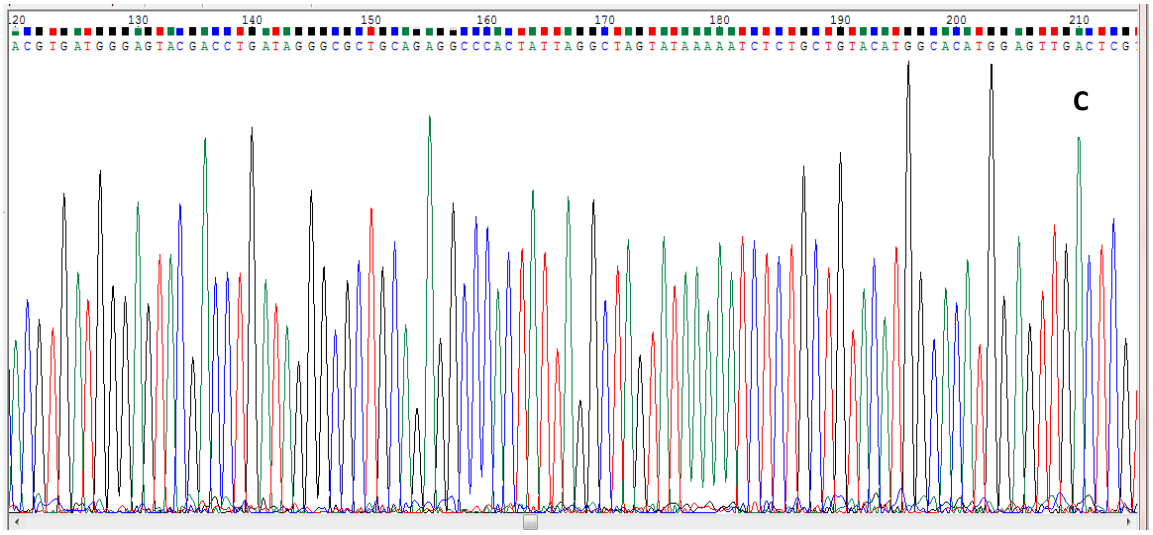


Figure 4.3: Chromatograms obtained after sequencing viewed on Chromas software. Figure A, B, C, D and E represents the chromatograms of E2 gene segments of sample HV1, HV2, HV3, MV and PC which shows the good quality of bases as the color at the top of each peak is full with less noise at the bottom.


A


B


C

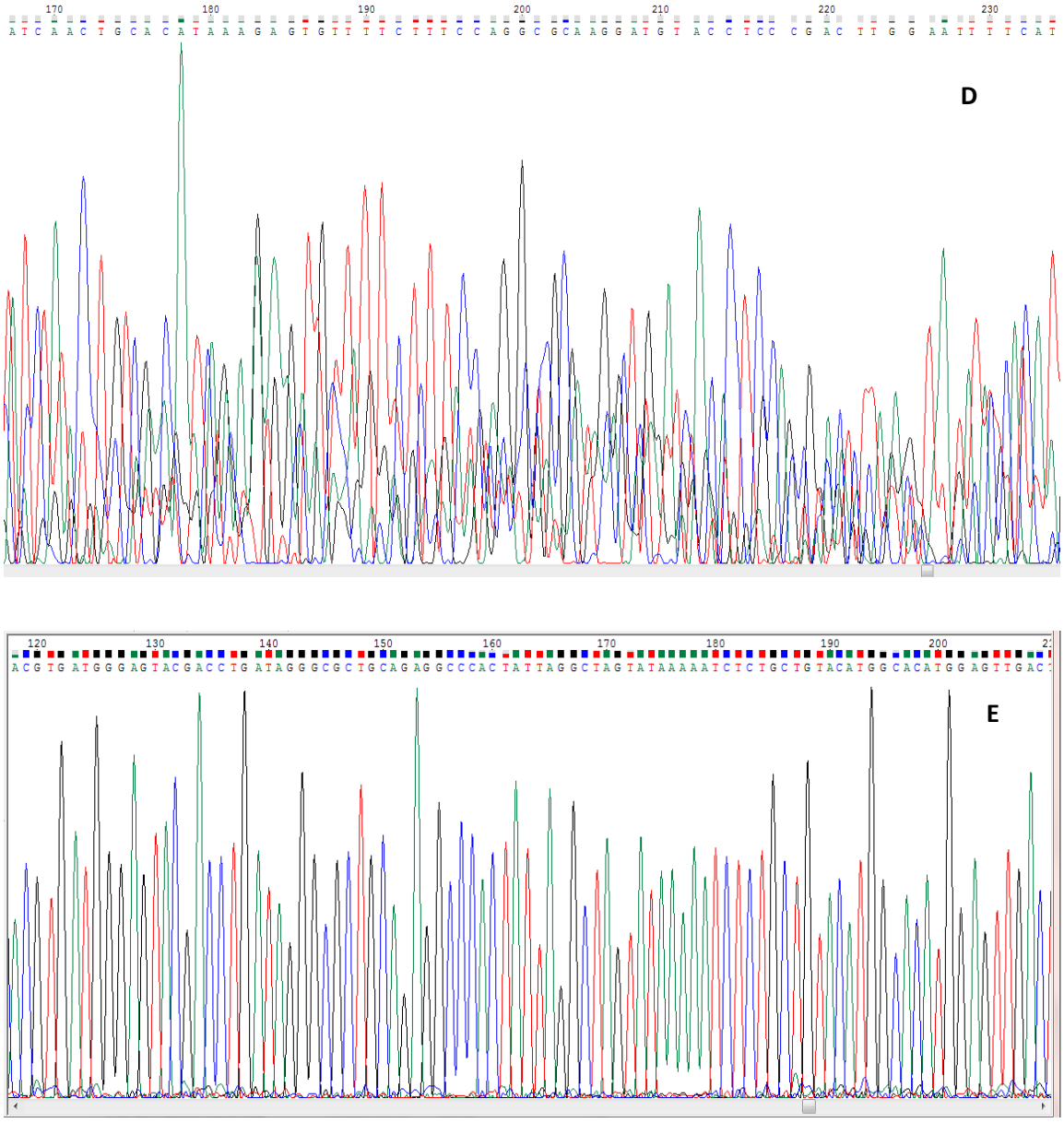


Figure 4.4: Chromatograms received after the sequencing of PCR product. Figure A, B, C, D and E represents the chromatograms of 5'NTR gene segments of samples HV1, HV2, HV3, MV and PC.

The contig of each sample was made by combining sequence obtained from forward and reverse sequencing with the help of BioEdit software. The contig was then used to BLAST in National Center for Biotechnological Information (NCBI). The blast result of E2 gene segment of the tested samples and positive control showed 97-99 percent (Figure 4.5) match with CSFV available in the NCBI database. In the case of blast result of 5'NTR gene segment, the tested samples showed 83-98 percent (Figure 4.6) match with CSFV available in NCBI database.

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Classical swine fever virus vaccine strain LPC/AHRI polyprotein gene, partial cds	481	481	99%	7e-132	98.53%	AY526732.1
Classical swine fever virus polyprotein gene, complete cds	464	464	99%	7e-127	97.43%	AF352565.1
Classical swine fever virus Nakhonpathom/NIAH818-3/01 envelope glycoprotein E2 gene, partial cds	448	448	99%	7e-122	96.32%	EU935426.1
Hog cholera virus envelope glycoprotein polyprotein gene, partial cds	448	448	99%	7e-122	96.34%	U35740.1
Classical swine fever virus Chonburi/NIAH132/01 envelope glycoprotein E2 gene, partial cds	442	442	99%	3e-120	95.96%	EU935422.1
Classical swine fever virus isolate HeN-AY polyprotein gene, partial cds	438	438	98%	4e-119	95.93%	GQ454793.1
Classical swine fever virus isolate Barra do Corda envelope glycoprotein E2 gene, partial cds	436	436	99%	1e-118	95.59%	KX431227.1
Classical swine fever virus Nakonnayok/NIAH1206/01 envelope glycoprotein E2 gene, partial cds	436	436	99%	1e-118	95.59%	EU935427.1
Pestivirus type 2 strain Alfort A19, complete genome	436	436	99%	1e-118	95.59%	U90951.1
Hog cholera virus (strain GPE-) complete sequence, encoding a polyprotein (complete cds)	436	436	99%	1e-118	95.59%	D49533.1
Classical swine fever virus, strain Alfort/187 complete genome	436	436	99%	1e-118	95.59%	NC_038912.1
Classical swine fever virus isolate CSF0741 envelope glycoprotein E2 gene, partial cds	431	431	99%	7e-117	95.22%	MK026454.1
Classical swine fever virus isolate CSF0738 envelope glycoprotein E2 gene, partial cds	431	431	99%	7e-117	95.22%	MK026451.1
Classical swine fever virus Chonburi/NIAH369-4/01 envelope glycoprotein E2 gene, partial cds	431	431	99%	7e-117	95.22%	EU935423.1
Classical swine fever virus strain Riems, complete genome	431	431	99%	7e-117	95.22%	AY259122.1
Hog cholera virus (Classical swine fever virus) "Chinese" strain (C-strain; EP 0 351 901 B1) encoding polyprotein	431	431	99%	7e-117	95.22%	Z46258.1
Hog cholera virus strain Riems, complete genome	431	431	99%	7e-117	95.22%	U45477.1
Hog cholera virus structural protein mRNAbn	431	431	99%	7e-117	95.22%	Z22525.1
Classical swine fever virus isolate CSF0739 envelope glycoprotein E2 gene, partial cds	425	425	99%	3e-115	94.85%	MK026452.1
Classical swine fever virus isolate KNU-1823-2 envelope glycoprotein E2 gene, partial cds	425	425	99%	3e-115	94.85%	MK121887.1

A

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Classical swine fever virus vaccine strain LPC/AHRI polyprotein gene, partial cds	492	492	100%	3e-135	99.26%	AY526732.1
Classical swine fever virus polyprotein gene, complete cds	475	475	100%	3e-130	98.16%	AF352565.1
Hog cholera virus envelope glycoprotein polyprotein gene, partial cds	459	459	100%	3e-125	97.07%	U35740.1
Classical swine fever virus isolate Barra do Corda envelope glycoprotein E2 gene, partial cds	448	448	100%	7e-122	96.32%	KX431227.1
Classical swine fever virus Nakonnayok/NIAH1206/01 envelope glycoprotein E2 gene, partial cds	448	448	100%	7e-122	96.32%	EU935427.1
Classical swine fever virus Nakhonpathom/NIAH818-3/01 envelope glycoprotein E2 gene, partial cds	448	448	100%	7e-122	96.32%	EU935426.1
Pestivirus type 2 strain Alfort A19, complete genome	448	448	100%	7e-122	96.32%	U90951.1
Hog cholera virus (strain GPE-) complete sequence, encoding a polyprotein (complete cds)	448	448	100%	7e-122	96.32%	D49533.1
Classical swine fever virus, strain Alfort/187 complete genome	448	448	100%	7e-122	96.32%	NC_038912.1
Classical swine fever virus isolate CSF0741 envelope glycoprotein E2 gene, partial cds	442	442	100%	3e-120	95.96%	MK026454.1
Classical swine fever virus isolate CSF0738 envelope glycoprotein E2 gene, partial cds	442	442	100%	3e-120	95.96%	MK026451.1
Classical swine fever virus Chonburi/NIAH369-4/01 envelope glycoprotein E2 gene, partial cds	442	442	100%	3e-120	95.96%	EU935423.1
Classical swine fever virus Chonburi/NIAH132/01 envelope glycoprotein E2 gene, partial cds	442	442	100%	3e-120	95.96%	EU935422.1
Classical swine fever virus strain Riems, complete genome	442	442	100%	3e-120	95.96%	AY259122.1
Hog cholera virus (Classical swine fever virus) "Chinese" strain (C-strain; EP 0 351 901 B1) encoding polyprotein	442	442	100%	3e-120	95.96%	Z46258.1
Hog cholera virus strain Riems, complete genome	442	442	100%	3e-120	95.96%	U45477.1
Hog cholera virus structural protein mRNAbn	442	442	100%	3e-120	95.96%	Z22525.1
Classical swine fever virus isolate HeN-AY polyprotein gene, partial cds	438	438	99%	4e-119	95.93%	GQ454793.1
Classical swine fever virus isolate CSF0739 envelope glycoprotein E2 gene, partial cds	436	436	100%	1e-118	95.59%	MK026452.1
Classical swine fever virus isolate KNU-1823-2 envelope glycoprotein E2 gene, partial cds	436	436	100%	1e-118	95.59%	MK121887.1

B

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Classical swine fever virus vaccine strain LPC/AHRI polyprotein gene, partial cds	486	486	99%	1e-133	98.90%	AY526732.1
Classical swine fever virus polyprotein gene, complete cds	470	470	99%	1e-128	97.79%	AF352565.1
Classical swine fever virus Nakhonpathom/NIAH818-3/01 envelope glycoprotein E2 gene, partial cds	453	453	99%	1e-123	96.69%	EU935426.1
Hog cholera virus envelope glycoprotein polyprotein gene, partial cds	453	453	99%	1e-123	96.70%	U35740.1
Classical swine fever virus Chonburi/NIAH132/01 envelope glycoprotein E2 gene, partial cds	448	448	99%	7e-122	96.32%	EU935422.1
Classical swine fever virus isolate HeN-AY polyprotein gene, partial cds	444	444	98%	9e-121	96.30%	GQ454793.1
Classical swine fever virus isolate Barra do Corda envelope glycoprotein E2 gene, partial cds	442	442	99%	3e-120	95.96%	KX431227.1
Classical swine fever virus Nakonnayok/NIAH1206/01 envelope glycoprotein E2 gene, partial cds	442	442	99%	3e-120	95.96%	EU935427.1
Pestivirus type 2 strain Alfort A19, complete genome	442	442	99%	3e-120	95.96%	U90951.1
Hog cholera virus (strain GPE-) complete sequence, encoding a polyprotein (complete cds)	442	442	99%	3e-120	95.96%	D49533.1
Classical swine fever virus, strain Alfort/187 complete genome	442	442	99%	3e-120	95.96%	NC_038912.1
Classical swine fever virus isolate CSF0741 envelope glycoprotein E2 gene, partial cds	436	436	99%	1e-118	95.59%	MK026454.1
Classical swine fever virus isolate CSF0738 envelope glycoprotein E2 gene, partial cds	436	436	99%	1e-118	95.59%	MK026451.1
Classical swine fever virus Chonburi/NIAH369-4/01 envelope glycoprotein E2 gene, partial cds	436	436	99%	1e-118	95.59%	EU935423.1
Classical swine fever virus strain Riems, complete genome	436	436	99%	1e-118	95.59%	AY259122.1
Hog cholera virus (Classical swine fever virus) "Chinese" strain (C-strain; EP 0 351 901 B1) encoding polyprotein	436	436	99%	1e-118	95.59%	Z46258.1
Hog cholera virus strain Riems, complete genome	436	436	99%	1e-118	95.59%	U45477.1
Hog cholera virus structural protein mRNAbn	436	436	99%	1e-118	95.59%	Z22525.1
Classical swine fever virus isolate CSF0739 envelope glycoprotein E2 gene, partial cds	431	431	99%	7e-117	95.22%	MK026452.1
Classical swine fever virus isolate KNU-1823-2 envelope glycoprotein E2 gene, partial cds	431	431	99%	7e-117	95.22%	MK121887.1

C

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Classical swine fever virus vaccine strain LPC/AHRI polyprotein gene, partial cds	459	459	100%	3e-125	97.42%	AY526732.1
Classical swine fever virus polyprotein gene, complete cds	442	442	100%	3e-120	96.31%	AF352565.1
Classical swine fever virus Nakhonpathom/NIAH818-3/01 envelope glycoprotein E2 gene, partial cds	431	431	100%	7e-117	95.57%	EU935426.1
Classical swine fever virus Chonburi/NIAH132/01 envelope glycoprotein E2 gene, partial cds	425	425	100%	3e-115	95.20%	EU935422.1
Hog cholera virus envelope glycoprotein polyprotein gene, partial cds	425	425	100%	3e-115	95.22%	U35740.1
Classical swine fever virus isolate Barra do Corda envelope glycoprotein E2 gene, partial cds	420	420	100%	1e-113	94.83%	KX431227.1
Pestivirus type 2 strain Alfort A19, complete genome	420	420	100%	1e-113	94.83%	U90951.1
Hog cholera virus (strain GPE-) complete sequence, encoding a polyprotein (complete cds)	420	420	100%	1e-113	94.83%	D49533.1
Classical swine fever virus, strain Alfort/187 complete genome	420	420	100%	1e-113	94.83%	NC_038912.1
Classical swine fever virus isolate HeN-AY polyprotein gene, partial cds	416	416	99%	2e-112	94.80%	GQ454793.1
Classical swine fever virus isolate CSF0741 envelope glycoprotein E2 gene, partial cds	414	414	100%	7e-112	94.46%	MK026454.1
Classical swine fever virus isolate CSF0738 envelope glycoprotein E2 gene, partial cds	414	414	100%	7e-112	94.46%	MK026451.1
Classical swine fever virus Nakonnayok/NIAH1206/01 envelope glycoprotein E2 gene, partial cds	414	414	100%	7e-112	94.46%	EU935427.1
Classical swine fever virus Chonburi/NIAH369-4/01 envelope glycoprotein E2 gene, partial cds	414	414	100%	7e-112	94.46%	EU935423.1
Hog cholera virus structural protein mRNAbn	414	414	100%	7e-112	94.46%	Z22525.1
Classical swine fever virus isolate CSF0739 envelope glycoprotein E2 gene, partial cds	411	411	99%	9e-111	94.42%	MK026452.1
Classical swine fever virus isolate KNU-1823-2 envelope glycoprotein E2 gene, partial cds	409	409	100%	3e-110	94.10%	MK121887.1
Classical swine fever virus isolate LOM(JJ-1602) polyprotein gene, partial cds	409	409	100%	3e-110	94.10%	KX954608.1
Classical swine fever virus strain Koslov clone Kos_4aa, complete genome	409	409	100%	3e-110	94.10%	KF977610.1
Classical swine fever virus strain Koslov clone Kos_3aa, complete genome	409	409	100%	3e-110	94.10%	KF977609.1

D

	Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
☐	Classical swine fever virus vaccine strain LPC/AHRI polyprotein gene, partial cds	481	481	100%	7e-132	98.53%	AY526732.1
☐	Classical swine fever virus polyprotein gene, complete cds	464	464	100%	7e-127	97.43%	AF352565.1
☐	Classical swine fever virus Nakhonpathom/NIAH818-3/01 envelope glycoprotein E2 gene, partial cds	448	448	100%	7e-122	96.32%	EU935426.1
☐	Hog cholera virus envelope glycoprotein polyprotein gene, partial cds	448	448	100%	7e-122	96.34%	U35740.1
☐	Classical swine fever virus Chonburi/NIAH132/01 envelope glycoprotein E2 gene, partial cds	442	442	100%	3e-120	95.96%	EU935422.1
☐	Classical swine fever virus isolate HeN-AY polyprotein gene, partial cds	438	438	99%	4e-119	95.93%	GQ454793.1
☐	Classical swine fever virus isolate Barra do Corda envelope glycoprotein E2 gene, partial cds	436	436	100%	1e-118	95.59%	KX431227.1
☐	Classical swine fever virus Nakonnayok/NIAH1206/01 envelope glycoprotein E2 gene, partial cds	436	436	100%	1e-118	95.59%	EU935427.1
☐	Pestivirus type 2 strain Alfort A19, complete genome	436	436	100%	1e-118	95.59%	U90951.1
☐	Hog cholera virus (strain GPE-) complete sequence, encoding a polyprotein (complete cds)	436	436	100%	1e-118	95.59%	D49533.1
☐	Classical swine fever virus, strain Alfort/187 complete genome	436	436	100%	1e-118	95.59%	NC_038912.1
☐	Classical swine fever virus isolate CSF0741 envelope glycoprotein E2 gene, partial cds	431	431	100%	7e-117	95.22%	MK026454.1
☐	Classical swine fever virus isolate CSF0738 envelope glycoprotein E2 gene, partial cds	431	431	100%	7e-117	95.22%	MK026451.1
☐	Classical swine fever virus Chonburi/NIAH369-4/01 envelope glycoprotein E2 gene, partial cds	431	431	100%	7e-117	95.22%	EU935423.1
☐	Classical swine fever virus strain Riems, complete genome	431	431	100%	7e-117	95.22%	AY259122.1
☐	Hog cholera virus (Classical swine fever virus) "Chinese" strain (C-strain; EP 0 351 901 B1) encoding polyprotein	431	431	100%	7e-117	95.22%	Z46258.1
☐	Hog cholera virus strain Riems, complete genome	431	431	100%	7e-117	95.22%	U45477.1
☐	Hog cholera virus structural protein mRNAbn	431	431	100%	7e-117	95.22%	Z22525.1
☐	Classical swine fever virus isolate CSF0739 envelope glycoprotein E2 gene, partial cds	425	425	100%	3e-115	94.85%	MK026452.1
☐	Classical swine fever virus isolate KNU-1823-2 envelope glycoprotein E2 gene, partial cds	425	425	100%	3e-115	94.85%	MK121887.1

E

Figure 4. 5: The blast result obtained from NCBI by blast of contig made using BioEdit. Figure A, B, C, D and E represents the blast result of E2 gene segment of HV1, HV2, HV3, MV and PC.

	Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
☐	Classical swine fever virus polyprotein gene, complete cds	475	475	98%	3e-130	98.87%	AF352565.1
☐	Classical swine fever virus VN91 genomic RNA, complete genome	464	464	100%	7e-127	97.77%	LC374604.1
☐	Classical swine fever virus strain CSFV-GZ-2009, complete genome	464	464	100%	7e-127	97.77%	HQ380231.1
☐	Classical swine fever virus strain Eystrup, complete genome	464	464	100%	7e-127	97.77%	AF326963.1
☐	Classical swine fever virus 39, complete genome	464	464	100%	7e-127	97.77%	AF407339.1
☐	Classical swine fever virus strain cF114, complete genome	464	464	100%	7e-127	97.77%	AF333000.1
☐	Classical swine fever virus strain Rovac, complete genome	459	459	100%	3e-125	97.40%	KJ873238.1
☐	Classical swine fever virus strain Koslov clone Kos_4aa, complete genome	459	459	100%	3e-125	97.40%	KF977610.1
☐	Classical swine fever virus strain Koslov clone Kos_3aa, complete genome	459	459	100%	3e-125	97.40%	KF977609.1
☐	Classical swine fever virus strain Koslov clone Kos_2aa, complete genome	459	459	100%	3e-125	97.40%	KF977608.1
☐	Classical swine fever virus strain Koslov clone Kos, complete genome	459	459	100%	3e-125	97.40%	KF977607.1
☐	Classical swine fever virus strain CSFV/1.1/dp/CSF0382/XXXX/Koslov, complete genome	459	459	100%	3e-125	97.40%	HM237795.1
☐	Classical swine fever virus strain JL1(06), complete genome	459	459	100%	3e-125	97.40%	EU497410.1
☐	Classical swine fever virus strain Shimen/HVRI, complete genome	459	459	100%	3e-125	97.40%	AY775178.2
☐	Classical swine fever virus isolate LK-VNIVViM, complete genome	453	453	100%	1e-123	97.03%	KM522833.1
☐	Classical swine fever virus strain C-ZJ-2008, complete genome	453	453	100%	1e-123	97.03%	HM175885.1
☐	Classical swine fever virus from India, complete genome	453	453	100%	1e-123	97.03%	EU857642.1
☐	Classical swine fever virus strain Thiverval, complete genome	453	453	100%	1e-123	97.03%	EU490425.1
☐	Classical swine fever virus isolate 4/7 P-2 5' UTR and polyprotein gene, partial cds	453	453	100%	1e-123	97.03%	EF051174.1
☐	Classical swine fever virus strain C/HVRI, complete genome	453	453	100%	1e-123	97.03%	AY805221.1

A

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Classical swine fever virus polyprotein gene, complete cds	193	193	79%	3e-45	83.72%	AF352565.1
Classical swine fever virus VN91 genomic RNA, complete genome	187	187	79%	2e-43	83.26%	LC374604.1
Classical swine fever virus strain CSFV-GZ-2009, complete genome	187	187	79%	2e-43	83.26%	HQ380231.1
Classical swine fever virus strain Eystrup, complete genome	187	187	79%	2e-43	83.26%	AF326963.1
Classical swine fever virus 39, complete genome	187	187	79%	2e-43	83.26%	AF407339.1
Classical swine fever virus strain cF114, complete genome	187	187	79%	2e-43	83.26%	AF333000.1
Classical swine fever virus strain BV-P 5' NTR and polyprotein gene, partial cds	187	187	79%	2e-43	83.26%	DQ314582.1
Classical swine fever virus strain Rovac, complete genome	182	182	79%	7e-42	82.79%	KJ873238.1
Classical swine fever virus strain Koslov clone Kos_4aa, complete genome	182	182	79%	7e-42	82.79%	KF977610.1
Classical swine fever virus strain Koslov clone Kos_3aa, complete genome	182	182	79%	7e-42	82.79%	KF977609.1
Classical swine fever virus strain Koslov clone Kos_2aa, complete genome	182	182	79%	7e-42	82.79%	KF977608.1
Classical swine fever virus strain Koslov clone Kos, complete genome	182	182	79%	7e-42	82.79%	KF977607.1
Classical swine fever virus isolate 5NCR/CSF/MZ/AIZ/348 Npro gene, partial cds	182	182	79%	7e-42	82.87%	JX975460.1
Classical swine fever virus strain CSFV/1.1/dp/CSF0382/XXXX/Koslov, complete genome	182	182	79%	7e-42	82.79%	HM237795.1
Classical swine fever virus strain C-ZJ-2008, complete genome	182	182	79%	7e-42	82.79%	HM175885.1
Classical swine fever virus strain Thiverval, complete genome	182	182	79%	7e-42	82.79%	EU490425.1
Classical swine fever virus strain JL1(06), complete genome	182	182	79%	7e-42	82.87%	EU497410.1
Classical swine fever virus strain Shimen/HVRI, complete genome	182	182	79%	7e-42	82.87%	AY775178.2
Classical swine fever virus strain C/HVRI, complete genome	182	182	79%	7e-42	82.79%	AY805221.1
Classical swine fever virus, complete genome	182	182	79%	7e-42	82.79%	AY663656.1

B

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Classical swine fever virus polyprotein gene, complete cds	628	628	99%	3e-176	97.81%	AF352565.1
Classical swine fever virus 39, complete genome	595	595	99%	3e-166	96.16%	AF407339.1
Classical swine fever virus isolate 5NCR/CSF/MZ/AIZ/348 Npro gene, partial cds	592	592	99%	4e-165	95.90%	JX975460.1
Classical swine fever virus strain cF114, complete genome	590	590	99%	1e-164	95.89%	AF333000.1
Classical swine fever virus VN91 genomic RNA, complete genome	584	584	99%	7e-163	95.62%	LC374604.1
Classical swine fever virus strain Koslov clone Kos_4aa, complete genome	584	584	99%	7e-163	95.62%	KF977610.1
Classical swine fever virus strain Koslov clone Kos_3aa, complete genome	584	584	99%	7e-163	95.62%	KF977609.1
Classical swine fever virus strain Koslov clone Kos_2aa, complete genome	584	584	99%	7e-163	95.62%	KF977608.1
Classical swine fever virus strain Koslov clone Kos, complete genome	584	584	99%	7e-163	95.62%	KF977607.1
Classical swine fever virus strain CSFV-GZ-2009, complete genome	584	584	99%	7e-163	95.62%	HQ380231.1
Classical swine fever virus strain CSFV/1.1/dp/CSF0382/XXXX/Koslov, complete genome	584	584	99%	7e-163	95.62%	HM237795.1
Classical swine fever virus from India, complete genome	584	584	99%	7e-163	95.62%	EU857642.1
Classical swine fever virus strain Eystrup, complete genome	584	584	99%	7e-163	95.62%	AF326963.1
Classical swine fever virus isolate 5NCR/CSF/MZ/AIZ/352 Npro gene, partial cds	580	580	99%	9e-162	95.36%	JX975461.1
Classical swine fever virus strain Rovac, complete genome	579	579	99%	3e-161	95.34%	KJ873238.1
Classical swine fever virus strain C-ZJ-2008, complete genome	579	579	99%	3e-161	95.34%	HM175885.1
Classical swine fever virus strain JL1(06), complete genome	579	579	99%	3e-161	95.34%	EU497410.1
Classical swine fever virus strain Shimen/HVRI, complete genome	579	579	99%	3e-161	95.34%	AY775178.2
Classical swine fever virus strain C/HVRI, complete genome	579	579	99%	3e-161	95.34%	AY805221.1

C

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Classical swine fever virus vaccine strain LPC/AHRI polyprotein gene, partial cds	53.6	53.6	42%	0.003	72.22%	gi|42560497|AY526732.1
Classical swine fever virus strain LPC/TWN E2 protein gene, partial cds	53.6	53.6	42%	0.003	72.22%	gi|50403915|AY571095.1
Kuraishia capsulata CBS 1993 uncharacterized protein (KUCA_T0000525500 1), partial mRNA	51.8	51.8	23%	0.011	81.36%	gi|1247157669|XM_022600433.1
Classical swine fever virus polyprotein gene, complete cds	46.4	46.4	30%	0.48	74.03%	gi|13605326|AF352565.1
Classical swine fever virus isolate SC15 glycoprotein E2 gene, partial cds	44.6	44.6	42%	1.7	70.37%	gi|1101656116|KT986036.1
Classical swine fever virus isolate SC8 glycoprotein E2 gene, partial cds	44.6	44.6	42%	1.7	70.37%	gi|1101656102|KT986029.1
Classical swine fever virus strain SWH, complete genome	44.6	44.6	42%	1.7	70.37%	gi|71084281|DQ127910.1
Classical swine fever virus Col7487-02 E2 glycoprotein (E2) gene, partial cds	44.6	44.6	42%	1.7	70.37%	gi|32187076|AY308864.1
Classical swine fever virus Col5704-02 E2 glycoprotein (E2) gene, partial cds	44.6	44.6	42%	1.7	70.37%	gi|32187067|AY308862.1
Classical swine fever virus isolate SC10 glycoprotein E2 gene, partial cds	43.7	43.7	42%	5.9	70.64%	gi|1101656106|KT986031.1

D

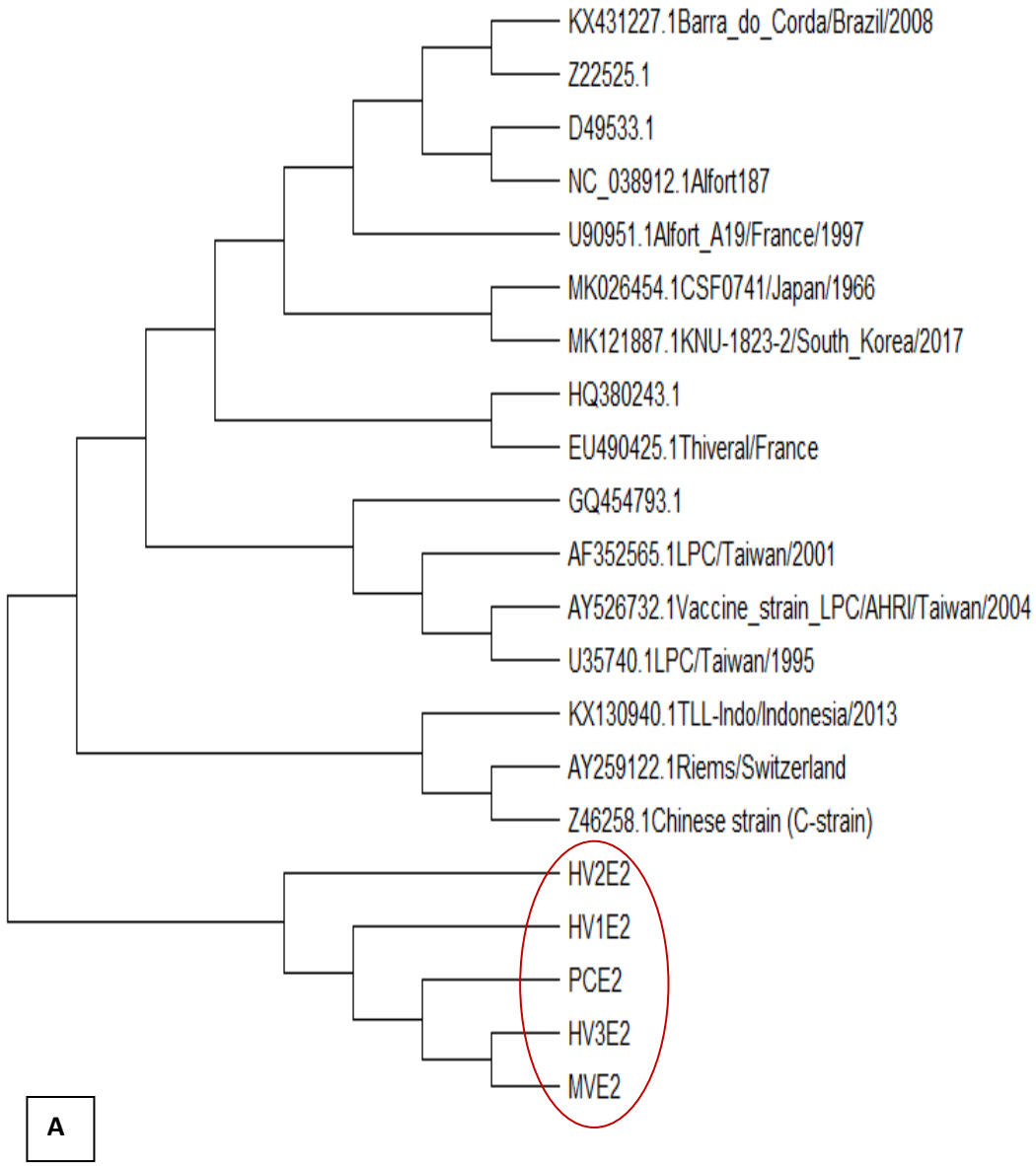
Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Classical swine fever virus polyprotein gene, complete cds	466	466	100%	2e-127	97.79%	AF352565.1
Classical swine fever virus VN91 genomic RNA, complete genome	455	455	100%	4e-124	97.05%	LC374604.1
Classical swine fever virus strain CSFV-GZ-2009, complete genome	455	455	100%	4e-124	97.05%	HQ380231.1
Classical swine fever virus strain Eystrup, complete genome	455	455	100%	4e-124	97.05%	AF326963.1
Classical swine fever virus 39, complete genome	455	455	100%	4e-124	97.05%	AF407339.1
Classical swine fever virus strain cF114, complete genome	455	455	100%	4e-124	97.05%	AF333000.1
Classical swine fever virus strain Rovac, complete genome	449	449	100%	2e-122	96.68%	KJ873238.1
Classical swine fever virus strain Koslov clone Kos_4aa, complete genome	449	449	100%	2e-122	96.68%	KF977610.1
Classical swine fever virus strain Koslov clone Kos_3aa, complete genome	449	449	100%	2e-122	96.68%	KF977609.1
Classical swine fever virus strain Koslov clone Kos_2aa, complete genome	449	449	100%	2e-122	96.68%	KF977608.1
Classical swine fever virus strain Koslov clone Kos, complete genome	449	449	100%	2e-122	96.68%	KF977607.1
Classical swine fever virus strain CSFV/1.1/dp/CSF0382/XXXX/Koslov, complete genome	449	449	100%	2e-122	96.68%	HM237795.1
Classical swine fever virus strain JL1(06), complete genome	449	449	100%	2e-122	96.68%	EU497410.1
Classical swine fever virus strain Shimen/HVRI, complete genome	449	449	100%	2e-122	96.68%	AY775178.2
Hog cholera virus strain HCLV, complete genome	449	449	100%	2e-122	96.68%	AF091507.1
Classical swine fever virus isolate LK-VNIVViM, complete genome	444	444	100%	9e-121	96.31%	KM522833.1
Classical swine fever virus strain C-ZJ-2008, complete genome	444	444	100%	9e-121	96.31%	HM175685.1
Classical swine fever virus from India, complete genome	444	444	100%	9e-121	96.31%	EU857642.1
Classical swine fever virus strain Thiverval, complete genome	444	444	100%	9e-121	96.31%	EU490425.1
Classical swine fever virus isolate 4/7 P-2 5' UTR and polyprotein gene, partial cds	444	444	100%	9e-121	96.31%	EF051174.1

E

Figure 4.6: The blast result obtained from NCBI by blast of contig made using BioEdit software. Figure A, B, C and D represents the blast result of 5'NTR gene segment of HV1, HV2, HV3, MV and PC.

4.5 Phylogenetic Analysis

The maximum likelihood phylogenetic tree constructed by aligning the sequences of tested samples, positive control and reference sequences from NCBI with ClustalW algorithm using MEGA-X program. Phylogenetic analysis on the basis of E2 gene segment (Figure) showed that the tested samples were closest to the positive control and other strains from NCBI such as Chinese strain (C-strain) followed by TLL-Indo, Riems C, Lapinized Philippines Coronel (LPC) and other strains and distantly related to AlfortA19 and Alfort187 from from France and Barra do Corda from Brazil. On the basis of 5'NTR gene segment (Figure), the samples were found to be closely related to the positive control and other CSFV strains from NCBI such as LPC strain, followed by virus with accession number AY805221.1, AF091507.1 , Chinese strain and others and distantly related to AlfortA19, Alfort187 and Thiverval strains.


KX431227.1Barra_do_Corda/Brazil/2008
Z22525.1
D49533.1
NC_038912.1Alfort187
U90951.1Alfort_A19/France/1997
MK026454.1CSF0741/Japan/1966
MK121887.1KNU-1823-2/South_Korea/2017
HQ380243.1
EU490425.1Thiveral/France
GQ454793.1
AF352565.1LPC/Taiwan/2001
AY526732.1Vaccine_strain_LPC/AHRI/Taiwan/2004
U35740.1LPC/Taiwan/1995
KX130940.1TLL-Indo/Indonesia/2013
AY259122.1Riems/Switzerland
Z46258.1Chinese strain (C-strain)
HV2E2
HV1E2
PCE2
HV3E2
MVE2
A

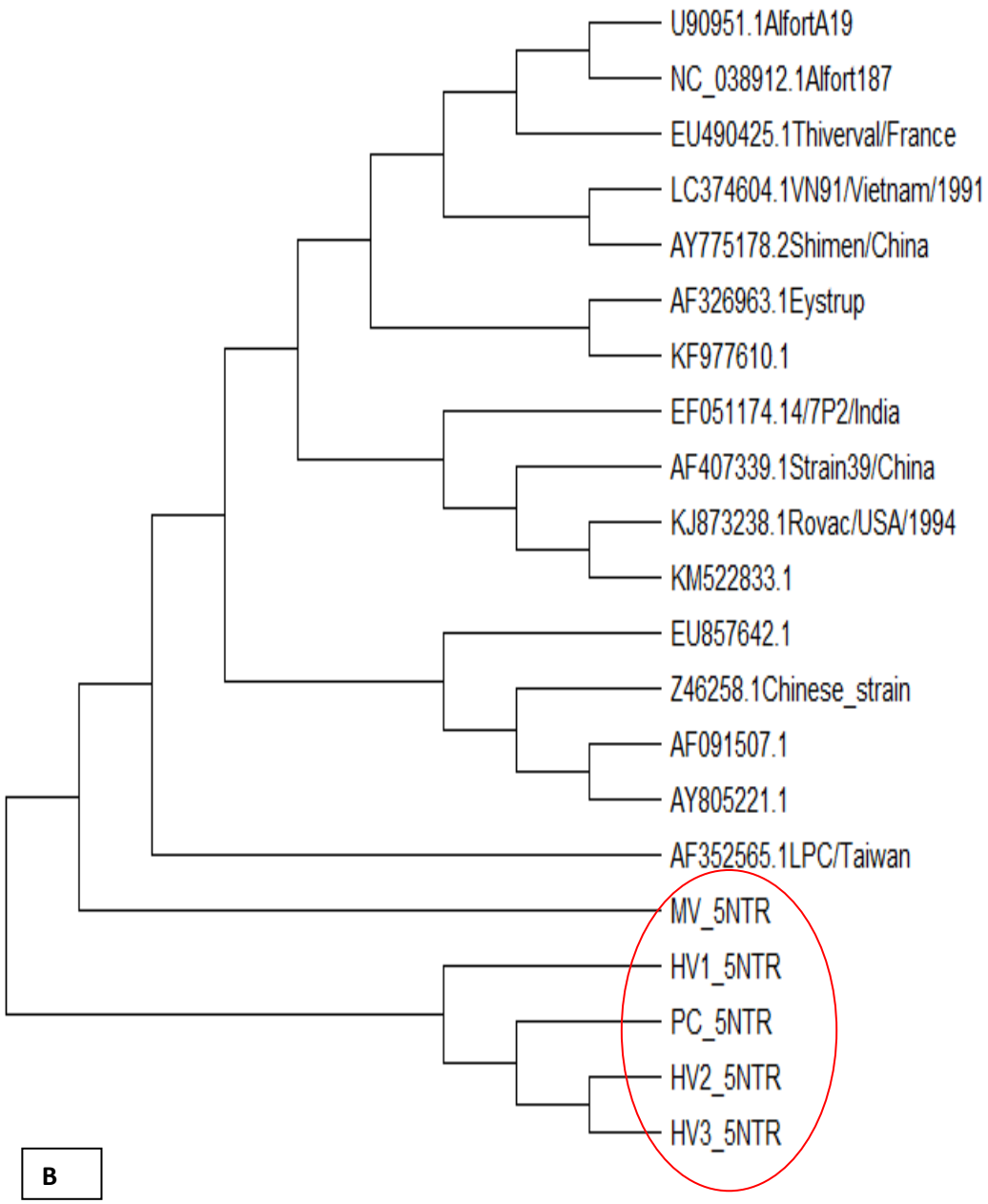


Figure 4.6: Phylogenetic tree (A) based on E2 gene segment and (B) based on 5'NTR gene segment. The tree was constructed using MEGA-X software for tested samples and 16 reference isolates from GenBank.

4.6 Detection of CSFV by real time RT-PCR

All the tested samples were found CSFV positive by real time RT-PCR. In the 2-step RT-qPCR, the targeted gene segment i.e NS5B was found to be amplified in all the tested

sample with different quantification cycle (Cq) (Figure-4.8). There was observed a single peak for individual samples in melting curve analysis (Figure-4.9).

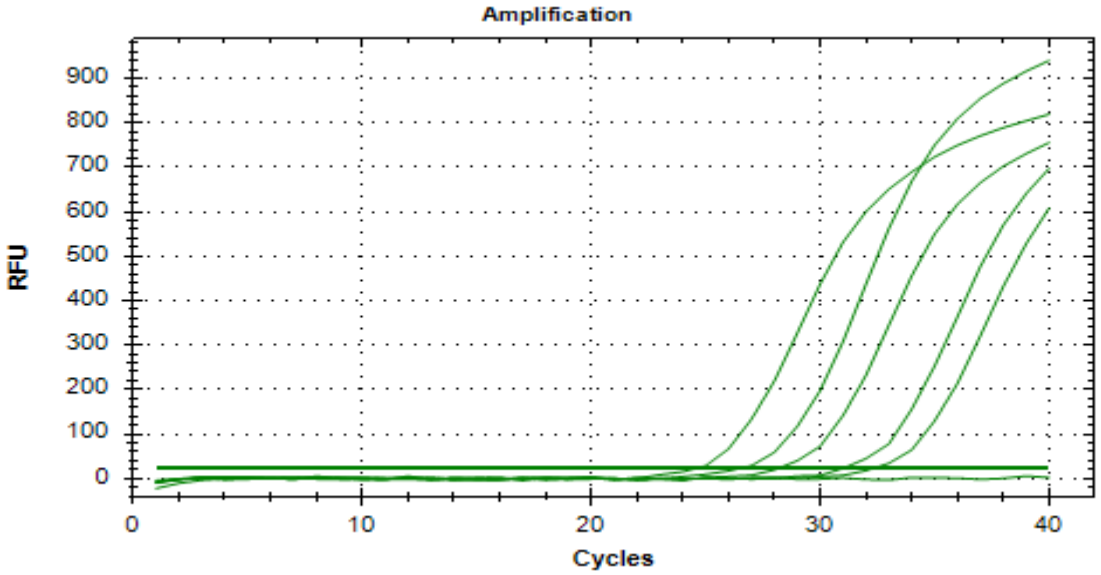


Figure 4. 7: Real time PCR curve showing amplification of all the samples

Table 4. 3: The table showing the Ct values of different samples in amplification of targeted gene segment by SYBR green real time PCR assay.

Sample	Ct	Ct Mean	Ct Std. Dev
NC		0.00	0.000
HV1	28.23	28.23	0.000
HV2	32.39	32.39	0.000
HV3	26.59	26.59	0.000
MV	31.06	31.06	0.000
PC	24.67	24.67	0.000

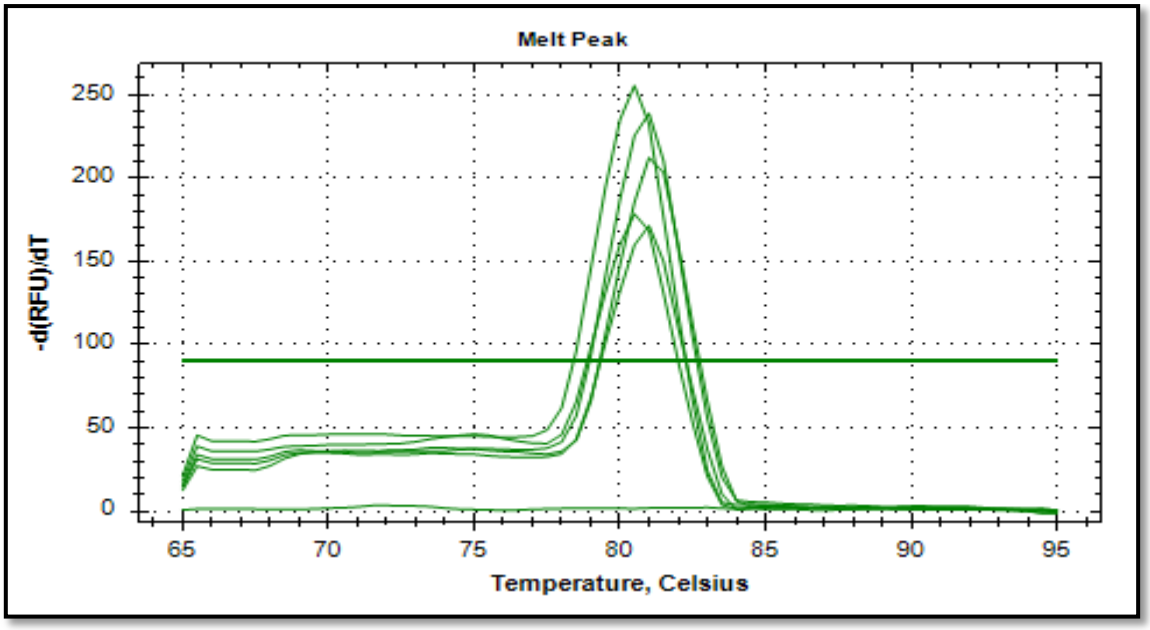


Figure 4. 8: The melt curve showing single peak per sample at 81◦C indicating absence of non-specific amplification.

Chapter V

DISCUSSION

Classical swine fever has been well known for decades with epidemics of disease in different parts of world and still occurring in Nepal. It causes disease in both domestic pigs and wild boars. The clinical diagnosis of the disease is not enough for veterinary practitioners to conform the disease as the symptoms of CSF disease similar with the symptoms of other infectious diseases. The CSF disease is of great concern for Nepal as it is agricultural country having about 66% people directly engaged in farming and livestock is an integral component of farming system. In Nepal, pig farming has played important role in poverty reduction by improving the economic and social condition of the poor, socially disadvantaged people and women. According to the report published by Department of Livestock Services, Ministry of Agriculture, Land Management and Cooperatives, Government of Nepal 2016/2017, the population of pigs in the country in 2016/17 is 1328036 and meat product (pork) in the same year is 24535 metric tons(Department of lovestock services, 2016/17). Nepal government has applied preventive strategy to fight against the highly contagious disease which can be devastating in pig farming if uncontrolled. Therefore Nepal government has produced vaccines against the CSFV within the country.

It is difficult to differentiate classical swine fever from other diseases only on the basis of high fever. The disease can be suspected on the basis of high fever and it should be confirmed by laboratory testing like virus isolation and molecular characterization of the causative agent. Now the fluorescent antibody techniques to antigen detection, use of ELISA for serology, development and application of monoclonal antibodies to the virus and molecular technology for epidemiological investigation have made significant advances in the laboratory diagnosis of CSFV infection(Edwards et al., 2000). With the application of molecular techniques now the changes in the virus population over time and in different environment conditions can be determined which can be use in epidemiology of the causative agent for assessing the origin of virus which can be used in prevention and control of the disease. This study was undertaken to isolate and characterize the CSFV from vaccine samples of Nepal.

In the present study three vaccine samples and one seed virus as positive control were taken from Hester Biosciences Nepal Private Limited and one vaccine sample from market which was produced by Central Biological Production Laboratory was taken. As the CSFV is RNA virus, RNA was extracted from all the vaccine samples and the concentration and purity of the RNA was determined. From the study, the RNA

concentration of PC was found to be highest with 135.15 ng/uL indicating there was high viral load as it is the seed vaccine vile used for vaccine development. The concentration of MV was found to be 24.05ng/uL indicating highest among tested samples. Whereas, the concentration of the RNA extracted from HV1, HV2 and HV3 was found to be less with 0.29, 2.58 and 1.75 ng/uL respectively. The lesser RNA concentration in tested samples might be due to dilution of the seed vaccine load by inoculating the virus load in live rabbit and addition of other substances such as skimmed milk and antibiotics during development of the vaccines. Talking about the purity of the extracted RNA; the RNA extracted from the MV and PC was found to be pure with A260/A280 ratios 2.13 and 2.03 respectively as the pure RNA should have the A260/A280 ratios around 2.1. Whereas the RNA extracted from the sample HV1, HV2 and HV3 was found to be protein contaminated indicated by the lower ratio of A260/A280 i.e. 0.37, 1.14, 0.94 respectively. Nucleic acids absorb UV light at 260nm due to the aromatic base moieties within their structure i.e. purines (thymine, cytosine and uracil) and pyrimidines (adenine and guanine). The protein contamination in samples HV1, HV2 and HV3 might be due to the composition of the vaccine; as there was use of skimmed milk in the vaccine development by Hester Biosciences Nepal Private Limited. Whereas in the case of positive sample it was a seed virus used for the development of the HV1, HV2 and HV3 in which there was no use of skimmed milk. In the case of the cDNA synthesized from the extracted RNA, the concentration of cDNA synthesized from the positive control was found to be highest as it was synthesized by using the RNA with highest concentration. The cDNA of all the tested samples were found to be pure enough as the 260/280 nm ratios of all the tested samples were around 1.8.

The presence of CSFV was confirmed by amplification of E2 and 5'NTR gene segments. There was observed required band size i.e 271 bp for both the targeted gene segments on 1.5% agarose gel by nested PCR amplification. So all the vaccine samples were found to be CSFV positive. The nested polymerase chain reaction has been widely used for CSFV detection as it is the best diagnostic tool for early detection of CSFV with high sensitivity(Dewulf et al., 2004). The E2, 5'NTR and NS5B have been targeted for PCR amplification of CSFV (Hofmann *et al*., 1994; Lowings*et al*., 1996; 1998 and Paton *et al*., 2000). In this study, E2 and 5'NTR regions were included. The 5'NTR region was taken for PCR amplification as it is the most conserved region among all the Pestiviruses. This region is the most favorite target for PCR for the diagnosis of CSF as the region is genetically stable(Paton et al., 2000). But this region has a disadvantage in phylogenetic analysis as it gives poorer resolusion between closely related isolates(Lowings, Ibata,

Needham, & Paton, 1996). Whereas the E2 region is best for considerable genetic variability and good discrimination between similar CSFV isolates(Paton et al., 2000).

The phylogenetic analysis of the tested vaccine samples were conducted from the sequencing results obtained. Based on the E2 and 5'NTR gene segments of the tested samples and reference strains from NCBI, the tested samples were found closest to different lipinized attenuated vaccine strains. The tested samples were found to be close to the sub group 1.1; which matched with the report by Gupta et al. in which the Indian lapinized CSFV was found to be member of subgroup 1.1 within group 1(Gupta et al., 2011). In Nepal, outbreak of CSF disease by 2.2 subgenotype has been reported(Postel et al., 2013). Though the tested vaccine samples were found to be close to subgenotype 1.1, it can be used for prevention of infection by the 2.2 subgenotype as previous reports have suggested that although C-strain belongs to subgenotype 1.1, it is able to induce protection against virulent CSFV of wide-range of genotypes; including the prevalent subgenotypes 2.1 and 2.2, and less prevalent subgenotypes 2.3 and 1.1 in China(Qiu, Shen, & Tong, 2006).

Phylogenetic analysis on the basis of E2 gene segment (Figure 4.6) showed that the tested samples were closest to Chinese strain (C-strain) followed by TLL-Indo, Riems C, Lapinized Philippines Coronel (LPC) and other strains. The Chinese strain is the most commonly used lapinized vaccine strain of CSFV in the world for immunization of pigs against CSFV, TLL-Indo is an Indonesian isolate isolated in 2013, Riems C and LPC are vaccine strains. Whereas they were found distantly related to AlfortA19 and Alfort187 from France and Barra do Corda from Brazil. But in the case of analysis on the basis of 5'NTR gene segment (Figure 4.7) revealed that the samples were found closely related to the LPC strain, followed by virus with accession number AY805221.1, AF091507.1, Chinese strain and others. The LPC strain is lapinized vaccine strains of CSFV used for immunizing pigs against CSFV, the strain with accession number AY805221.1 is a member of sub genotype 1.1 isolated from China, strain with accession number AF091507.1 is hog cholera lapinized virus (HCLV) strain. Whereas they were found distantly related to AlfortA19, Alfort187 and Thiverval strains. From the phylogenetic analysis on the basis of E2 gene segment and 5'NTR gene segment, the tested samples were found closely related to the attenuated lapinized vaccine strains of CSFV; namely C-strain, LPC, HCLV and Riems C. These strains are most widely used in the world for immunizing pigs against CSFV(Pan et al., 2008). These findings were found to be similar to the report of Wong et al. in 2001 where full length cDNA of CSFV LPC vaccine strain was found to be closest to Chinese, Riems, HCLV, Alfort/187 and Brescia(Wong, Peng,

Liu, & Chang, 2001). The 5'NTR of Indian lapinized CSF vaccine strain has also shown the similar result(Gupta et al., 2011). Whereas the Alfort strains and Thiverval strain from France and Barra do Corda from Brazil were found to be distantly related to the tested samples.

The SYBR green based real time RT-PCR conducted also showed the tested vaccine samples were CSFV positive. The targeted NS5B gene segment of all the tested samples were amplified with Cq values from 24 to 32. The different Cq values for different samples indicated presence of different initial copy numbers of the targeted templates. The lower Cq value indicates presence of higher copy number of the targeted template. On comparing the Cq values of the tested samples, the Cq value of the positive sample was found to be lowest i.e. 24.67 indicating there was the highest number of viral RNA followed by the number of viruses in the vaccine vile than the rest of the tested samples. From the melt curve analysis, we concluded that the targeted gene segment was amplified correctly in all the samples as there was observed a single peak for individual samples. The inclusion of post-amplification melting curve analysis has been reported as a simple, straight forward way to check SYBR Green based reactions for any artefacts as well as to ensure specificity of the reaction(Kong, Omar, Bejo, Ideris, & Tan, 2009; Martinez et al., 2008; Tam, Clavijo, Engelhard, & Thurmond, 2009). So we can say that there was absence of nonspecific products and primer dimers which can reduce the amplification efficiency and ultimately the accuracy of the data.

Chapter VI

SUMMARY

This study was undertaken with the objectives of detection of CSFV in vaccine samples of Nepal and their molecular characterization. A total of five vaccine samples i.e three vaccine samples and one seed vaccine sample from Hester Biosciencess Nepal private Limited and one vaccine sample from market were taken. The RNA was extracted from the samples and found in good concentration and purity. The RNA thus extracted was used for cDNA synthesis which was further used in nested PCR amplification and SYBR green based real time PCR. All the tested samples were found positive by the Reverse Transcriptase nested PCR which yielded the desired PCR product of 271 bp for both E2 and 5'NTR gene segments. On the basis of the sequence obtained from the sequencing of the amplified PCR products, phylogenetic analysis was conducted for both E2 and 5'NTR segment. From phylogenetic analysis based on both the gene segments, it was found that the tested vaccine samples were found close to the sub group 1.1 and lapinized attenuated vaccine strains namely C-strain, LPC, HCLV and Riems C. The real time RT-PCR was also conducted successfully for the amplification of NS5B gene segment and gave the positive result for all the tested vaccine samples.

Chapter VII

CONCLUSION

From this work, it was concluded that all the tested vaccine samples are CSFV positive. The CSFV was detected in all the tested samples by RT-nPCR and RT-qPCR. The detected virus were found to be close to sub group 1.1. The phylogenetic analysis performed on the basis of sequence of both E2 and 5'NTR revealed that the tested vaccine samples are closest to the other vaccine strains such as C-strain, LPC, HCLV and Riems. So the vaccine can be used for the immunization of pigs against CSF disease. There is only one serotype of CSFV, there are 10 subgenotypes within 3 genotypes. The tested vaccine samples that found to be close to subgenotype 1.1 can be used for prevention of infection of all 3 genotypes and 10 subgenotypes as suggested by previous reports (Qiu et al., 2006). All the samples were also found positive with SYBR green based RT-qPCR which amplified the NS5B gene segment of the vaccine virus.

LIMITATIONS OF STUDY

- There is only one type of vaccine available in Nepali market (produced by Central Biological Production Laboratory) so the sample size was small and comparative study of different types of vaccines was not possible.
- There was not labeled sufficient information regarding the batch of the vaccine on the vial; so the batch wise comparison to see the changes in the genomics of the vaccine virus in different batch was not possible.
- We did not get the vaccine sample with known concentration of the virus so we were unable to determine the concentration of the virus in the tested samples.
- We were unable to get the CSF disease samples collected and stored from different outbreaks in past from FMD Laboratory, so we could not compare the disease causing strain and used vaccine strain in Nepal.
- The most of the time of research was consumed for getting the chemicals and reagents required for the work and sequencing of the PCR product as we have to depend on foreign countries for these purposes.

RECOMMENDATION

- Comparative study between the virus used in vaccine of Nepal and CSF disease causing virus in Nepal can be done in collaboration with FMD Laboratory for the disease samples.
- The vaccine strains found in this work can be compared with other vaccine strains if available in future in Nepal.
- Any new outbreak of CSF can be checked whether the outbreak is due to failure of the available vaccine strain or due to vast diverse virus strain from the vaccine strain.

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APPENDIX

During lab work at CDBT

Virology team with international delegates, CDBT