MOLECULAR AND SEROLOGICAL DIAGNOSIS OF JAPANESE ENCEPHALITIS IN PIGS

Thesis

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Dedicated to....

I.V.R.I.

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Certificate

This is to be certified that the research work embodied in this thesis entitled "Molecular and Serological Diagnosis of Japanese Encephalitis in Pigs" *submitted by* Dr. Mohan H.V., Roll No. 1298, *for the award of* Doctor of Philosophy *Degree in* Veterinary Public Health *at Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate himself under my supervision and guidance.*

It is further certified that Dr. Mohan H.V., Roll No. 1298, *has worked for more than 30 months in the Institute and has put in more than 300 days attendance under me from the date of registration for the* Doctor of Philosophy *Degree in this Deemed University, as required under the relevant ordinance.*

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Certificate

We the undersigned members of Advisory Committee of **Dr. Mohan H.V.,** *Roll No. 1298, a candidate for the degree of* **Doctor of Philosophy** *with the major discipline* **Veterinary Public Health**, *agree that the thesis entitled* **"Molecular and Serological Diagnosis of Japanese Encephalitis in Pigs"** *may be submitted in partial fulfillment of the requirement for the degree.*

We have gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented for the award of **Doctor of Philosophy** *Degree of this Institute.*

It is further certified that the candidate has completed all the prescribed requirements governing the award of **Doctor of Philosophy** *Degree of the Deemed University, Indian Veterinary Research Institute, Izatnagar.*

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(Mohan, H.V.)

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ABBREVIATIONS

AES	:	Acute Encephalitic Syndrome
AP61	:	*Aedes pseudoscutellaris*
BLAST	:	Basic Local Alignment Search Tool
C	:	Capsid
C6/36	:	Aedes albopictus clone
$CaCl_2$	:	Calcium Chloride
CFR	:	Case Fatality Rate
CSF	:	Cerebrospinal Fluid
Ct	:	Threshold cycle
cDNA	:	Complementary DNA
DALYs	:	Disability Adjusted Life Years
DEPC	:	Diethylpyrocarbonate
dNTP	:	Deoxyribonucleoside Triphosphate
ssRNA	:	Single-stranded Ribonucelic acid
DTT	:	Dithiothreitol
DW	:	Distilled Water
E	:	Envelope
EDTA	:	Ethylene Diamine Tetra-acetic acid
EIA	:	Enzyme Immunoassay
IFT	:	Immunofluorescence Technique
ELISA	:	Enzyme Linked Immunosorbant Assay
Fig	:	Figure
HI	:	Haemagglutination inhibition
HRPO	:	Horse Raddish Peroxidase
H_2O_2	:	Hydrogen Peroxide
H_2SO_4	:	Sulphuric acid
IgG	:	Immunoglobulin G
IgM	:	Immunoglobulin M
IVRI	:	Indian Veterinary Research Institute
JE	:	Japanese Encephalitis
JEV	:	Japanese Encephalitis Virus
LB	:	Luria Bertani
M	:	Membrane
MAb	:	Monoclonal Antibody
Mac-ELISA	:	IgM Capture ELISA
$MgCl_2$	:	Magnesium Chloride

M-MuLV	:	Moloney Murine Leukemia Virus
MVEV	:	Murray Valley Encephalitis Virus
NASBA	:	Nucleic acid sequence-based Amplification
NCBI	:	National Center for Biotechnology Information
NFW	:	Nuclease Free Water
NS	:	Non-structural Proteins
OPD	:	O-Phenylenediamine Dihydrochloride
PBMC	:	Peripheral Blood Mononuclear Cells
PBS	:	Phosphate Buffered Saline
PBST	:	Phosphate Buffered Saline with Tween-20
PCR	:	Polymerase Chain Reaction
PrM	:	Premembrane
PRNT	:	Plaque Reduction Neutralization Test
qRT-PCR	:	Quantitative Reverse Transcriptase PCR
RE	:	Restriction Endonuclease
RNA	:	Ribonucleic acid
RT-LAMP	:	Reverse Transcriptase Loop-mediated Isothermal Amplification
RT-PCR	:	Reverse Transcriptase Polymerase Chain Reaction
SMP	:	Skimmed Milk Powder
SLEV	:	St. Louis Encephalitis Virus
Taq	:	*Thermous aquaticus*
TAE	:	Tris Acetic acid EDTA
TBEV	:	Tick Borne Encephalitis Virus
TE	:	Tris EDTA
Tm	:	Melting temperature
UK	:	United Kingdom
USA	:	United States of America
UV	:	Ultraviolet
WHO	:	World Health Organization
WNV	:	West Nile Virus
YFV	:	Yellow Fever Virus

List of Measurements

bp	:	Base pair
°C	:	Degree Celsius
µg	:	Microgram
µl	:	Microlitre
%	:	Percentage
g	:	Gram
h	:	Hour
kb	:	Kilobase
kDa	:	Kilodalton
L	:	Litre
M	:	Molar
mg	:	Milligram
min	:	Minute
ml	:	Milliliter
mM	:	Millimole
ng	:	Nanogram
pmol	:	Picomole
rpm	:	Revolutions per minute
sec	:	Second
U	:	Unit
V	:	Volt

CONTENTS

Introduction

1 INTRODUCTION

Japanese encephalitis (JE) is a vector-borne viral disease that occurs in South Asia, Southeast Asia, East Asia, and the Pacific (Solomon, 2006). Japanese encephalitis virus (JEV) belongs to genus Flavivirus of family *Flaviviridae* and is transmitted between animals and human host by *Culex* sp. mosquitoes. JEV causes encephalitis in humans as well as in horses and abortion in pigs. The related neurotropic flaviviruses across the globe are : Equine encephalitis virus and St. Louis encephalitis virus in North America, West Nile virus in Africa and the Middle East, Murray Valley encephalitis virus in Australia, Roccio virus in South America and Tick borne encephalitis virus (TBEV) in Russia. These viral encephalitides share many virological, epidemiological and clinical features (Solomon, 2004). JE is an emerging viral disease having international importance because it is invading the previously non-endemic areas. From Asia, it has stretched to Papua New Guinea and Torres Strait of northern Australia (Hanna *et al.*, 1996; Mackenzie *et al.*, 1997). Molecular studies have suggested that all the flaviviruses originated from a common ancestor about 10,000–20,000 years ago and are rapidly evolving to fill the ecological niches. JE was first recognized in India in 1955 and since then major outbreaks from different parts of the country have been reported periodically (Dutta *et al.*, 2010).

The Japanese encephalitis virus (JEV) is a neurotropic flavivirus that affects the central nervous system, causing extensive damage that may lead to fatality in about one third of patients. Half of the survivors suffer from severe neuropshychiatric sequelae. With nearly 3 billion people living under the current JE-endemic region, recurring incidents of epidemic are being reported at regular intervals. Japanese encephalitis virus (JEV) has been implicated in periodic

outbreaks of human encephalitis cases in different countries of Asia and pose a major public health problem (Ghosh and Basu, 2009; Dutta *et al.*, 2010). In India, epidemiological data indicate prevalence of JEV in large parts of the country and the virus appears to be spreading to areas hitherto considered to be free of JEV activity (Banerjee, 1988). Rapid globalization, population explosion, changes in global climatic condition, industrialization and deforestation, all seem to correlate with the spread of the virus into newer territories. Today, with approximately three billion people living in the JE endemic region, there are estimated 35,000–50,000 cases and 10,000–15,000 deaths annually, thereby making JE one of the most dreaded vector-borne viral encephalitis in the world (Tsai, 2000; Solomon *et al.*, 2003). Since its first epidemic in 1935 in Japan, the infection is widespread throughout large parts of Asia and it has become an emerging disease of public health significance in South-East Asia, Indian subcontinent and Pacific (Solomon *et al.*, 2003). Globally, its occurrence is endemic in parts of China, India, Korea, Japan, South East Russian Federation, Islands in the Torres strait Australia, Nepal, Thailand, Vietnam, Cambodia, Phillipines, Taiwan, Indonesia, Malaysia and Srilanka. Approximately 60% of the world's population lives in these endemic regions. It has recurred annually in Japan and China with small outbreaks to more than 10,000 cases per year and case fatality rate (CFR) of 10% (Acha and Szyfrez, 2003). It is primarily a disease of children (5-15 years) with neurological sequel and death. It causes acute encephalitis with fatality rates ranging from 20% to as high as 50% (Burke and Leake, 1988; Vaughan and Hoke, 1992; Kaur and Vrati, 2003).

The JEV exists in a zoonotic transmission cycle among mosquitoes, pigs, bats, and water birds belonging to the family Ardeidae (cattle egrets and pond herons). Humans become infected when bitten by an infected mosquito and are a dead-end host because of low viremia, preventing the virus from being transmitted further (Solomon, 2004). The major mosquito vectors of JEV vary in different geographic regions; the most common are those of the *Culex* genus (Solomon, 2006; GourieDevi *et al*., 1995). Pigs are the main contributors in the transmission cycle with respect to human infection, because these animals often stay close to human dwellings. Ardeid birds are important maintenance hosts. JEV antibodies were detected in bats, revealing that bats can be a part of the JEV transmission cycle (Cui *et al.*, 2008). Vertical transmission of JEV in mosquitoes probably explains the "overwintering" of virus

between epidemics (GourieDevi *et al.*, 1995). JEV infection in domestic animals and other vertebrate species such as equines does not result in high viremias; thus, they are not expected to transmit the virus to humans. Amphibians and reptiles can be infected experimentally, but their role in overwintering and maintenance in the environment is not known (Mackenzie *et al.*, 2004; Solomon, 2006).

The antibody to the JEV in different animals provides an estimate of degree of exposure and susceptibility of respective species to JEV infection. A high prevalence of JEV antibodies has been documented in pigs, horses and birds and to a lesser extent in cattle, sheep, dogs and monkeys. Pigs and ardeid birds are the most important hosts for maintenance, amplification and spread of JEV. Pigs are the main component in the transmission cycle with respect to human infection whereas herons, egrets and other ardeid birds are important maintenance hosts (Erlanger *et al.*, 2009). JEV infected animals and mosquitoes generally remain asymptomatic, although fatal encephalitis occurs in horses and fetal wastage occurs in swine (Burke and Leake, 1988). JEV effects in infected domestic animals have lead to the development of animal vaccines. The domestic animals can get infected but show no evidence of viremia. Rodents appear to be unimportant hosts (Scherer *et al.*, 1959a).

Swine plays important role as an amplifying host in disease transmission. JEV infects number of animals such as swine, horse, dog, chicken, duck and reptiles in nature (Murphy *et al.*, 1999). Horses infected with JEV may develop fatal encephalitis with anorexia, lethargy and fever, whereas infection in swine is generally inapparent except stillbirths, abortion and aspermia respectively in infected sows and boars. It is enzootic in Asia and most commonly associated with rice growing fields in the rural areas where water lodging and irrigation system provides healthy environment for mosquito breeding. Two transmission pattern of JE exist in nature, an enzootic/endemic pattern in the tropical areas with seasonal outbreak in summer. The epidemiological pattern and virus activity is greatly influenced by climate, geography and immune status of the host population (Gubler, 2002).

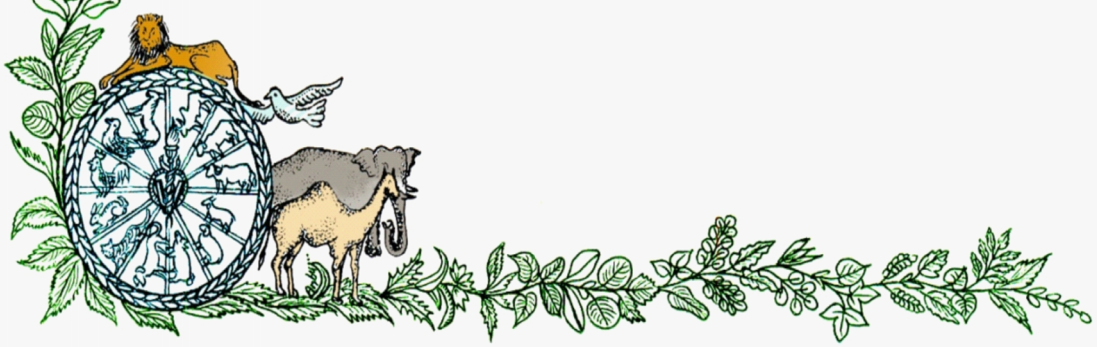
The laboratory diagnosis routinely used for JEV infection is based on four basic types of assays: serology, virus isolation, immunocytochemistry, and molecular techniques. Serologically, JEV infection can be detected by immunoglobulin M (IgM) and IgG capture enzyme-linked immunosorbent assay (ELISA) (Burke *et al.*, 1982; Endy and Nisalak, 2002;

Solomon *et al.,* 1998). However, confirmation and typing of virus are based on demonstration of fourfold or greater increase in the virus specific neutralizing antibody titer by plaque reduction neutralization (PRNT) assay with several flaviviruses. Virus isolation from clinical and surveillance samples has generally been unsuccessful, owing to the low level of transient viremia associated with the disease process, and also requires viable virus in samples. Patients with JE present vivid signs of acute encephalitic syndrome (AES). One of the recent advances in the diagnostics is the use of molecular techniques such as polymerase chain reaction (PCR), Real-time PCR, reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) etc. The reverse transcription polymerase chain reaction (RT-PCR) and Real-time RT-PCR has been reported by several workers for quick and sensitive detection of JEV RNA from clinical samples of human and animals (Shirato *et al.,* 2003; Yang *et al.,* 2005; Parida *et al.,* 2006a; Sapkal *et al.,* 2007; Santhosh *et al.,* 2007; Saxena *et al.,* 2009).

By keeping the above facts in view, there is an urgent need to know the prevalence of Japanese encephalitis in case of pigs in endemic regions of India. Therefore, the present study was carried with following objectives,

1. **To standardize reverse transcriptase-PCR (RT-PCR) and Real-time RT-PCR assay for the rapid detection of Japanese encephalitis in pigs.**
2. **To study the prevalence of JEV in pigs by Indirect enzyme linked immunosorbent assay, RT-PCR and Real-time PCR assay.**

Review of Literature



2 REVIEW OF LITERATURE

2.1 History

Japanese encephalitis was recognized in horses and humans as early as 1871. In 1924 a severe epidemic was reported from Japan; a filterable agent was extracted from human brain and passed to rabbits, although the agent could not be characterized. Every 10 years, major epidemics were reported in Japan affecting over 6000 patients (Miyake, 1964). In 1935, JE virus was isolated from human brain in Tokyo, Japan, and its virological and serological prototype, Nakayama strain, was established. The JEV was also isolated from the brain of a sick horse in 1937. Mosquito transmission of JEV was suspected in the early 1930s and JEV was isolated from *Culex tritaeniorrhynchus* by Mitamura and his colleagues (Mitamura *et al.,* 1936). The role of pigs and birds as reservoir in the transmission of JEV was established in 1959 (Buescher and Schere, 1959a).

In India, JE as a disease was first reported in 1955 when clinical cases were detected in Vellore and Pondicherry in southern India. The virus was however, not recovered from humans until 1958, when three isolations were made from the brain tissue of infected persons. Until early 1970s, the disease was reported only from southern India with periodic focal reports of its occurrence. A major outbreak resulting in 42.6% case-fatality rate was reported from Bankura district of West Bengal in 1973. Subsequently, the disease spread to other states and caused a series of outbreaks in different parts of the country. In 1978, cases were reported from 21 states and union territories, and from then onwards till 2007 there have been 1,03,389 reported cases of JE in India that has led to 33,729 deaths (Dhillon and Raina,

2008). Approximately 597,542,000 people in India live under the JE-endemic regions and there are 1500–4000 reported cases every year (Kabilan *et al.*, 2004a, b). These figures are based on total reported cases and it is quite possible that several cases may go unreported and hence the actual magnitude of the threat of JE may be considerably higher both in the Indian as well as the global context.

2.2 Japanese encephalitis virus

Japanese encephalitis virus (JEV) belongs to genus Flavivirus of family *Flaviviridae*. The JE virion consists of a single strand of positive-sense RNA of around 11 kilobase, inside a nucleocapsid and is surrounded by a glycoprotein-containing envelope. The RNA comprises a short 52 untranslated region (UTR), a longer 32 UTR and a single open reading frame between them. It codes for a single polyprotein, which is translationally and post-translationally cleaved by viral and host proteases into three structural proteins (core-C, premembrane-PrM and envelope-E), and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The C protein (12–14 kDa) is highly basic and combines with the RNA to form the nucleocapsid (Kaur and Vrati, 2003). The prM is closely associated with the E protein, forming a heterodimer and is thought to act as a 'chaperone' to it, impairing its function until after virion release. Immediately prior to virion release, the prM protein (18–19 kDa) is cleaved to its mature M protein (8–9 kDa) form. This allows the formation of E protein homodimers, which are thus 'activated'. The prM protein of JEV contains a single N-linked glycosylation site, which is highly conserved among the JEV strains. Researches indicated that this highly conserved N glycosylation motif in prM is crucial for multiple stages of JEV biology: prM biogenesis, virus release and pathogenesis (Kim *et al.*, 2008). The E protein is the largest structural protein (53–55 kDa), with up to two potential gylcosylation sites. It is the major target for the humoral immune response and is thought to be important for viral entry into host cells. It is worth mentioning that low pH is extremely important for viral entry into the cell to trigger viral membrane fusion with host endosomal membrane, thereby releasing the nucleoplasmid in the cytosol (Dutta *et al.*, 2010).

2.3 Genotypes

Phylogenic studies of a number of JEV isolates from different geographic areas using limited nucleotide sequencing in the highly variable PrM gene suggested that there are at least

5 JEV genotypes. These findings were confirmed using E gene sequencing (Chen *et al.*, 1990, 1992; Ni and Barrett, 1995; Paranjpe and Banerjee, 1996; Williams *et al.*, 2000). Most virus strains of genotype I were isolated from Northern Thailand, Cambodia and Korea; Genotype II from Southern Thailand, Indonesia, Malaysia and Australia; genotype III from areas of Asia that are largely temperate such as Japan, Korea, China, Taiwan, the Philippines, India and Sri Lanka; genotype IV from Indonesia; and genotype V from Malaysia (Chen *et al.*, 1990, 1992; Williams *et al.*, 2000; Uchil and Satchidanandam, 2001). Most isolates including the Nakayama strain belong to genotype III which is the most widely distributed genotype and the only genotype isolated from the Indian subcontinent. Genotypes I and III are principally distributed in temperate epidemic areas and genotypes II and IV in tropical endemic regions. The ancestor of JEV is probably an Asian virus and may have evolved in past 300 years (Mackenzie *et al.*, 2004).

2.4 Evolution and spread of JEV

The phylogenetic studies suggest that JEV spread from Southeast Asia to northern and eastern areas. Genotypes IV and V are the most divergent genotypes and may represent the oldest lineages. The clinical significance of these findings in humans is yet to be proven. The origin, evolution and spread of flavivirus have been investigated by detailed genomic sequence analysis and calculating base substitution rates using sequences from NS5, NS3, or E genes or from complex genomic equences (Gubler, 1997, 2002). The results have shown that tick-borne and mosquito-borne flaviviruses constitute two distinct separate evolutionary lineages (Kuno *et al.*, 1998; Billoir *et al.*, 2000). Taking the phylogenic data together with the biological properties of different flaviviruses, it has been suggested that flavivirus genus might have evolved from an ancestral virus in Africa about 10,000 years back (Billoir *et al.*, 2000; Gould *et al.*, 2004). The tick borne lineage is believed to have diverged about 3000 years back followed by the mosquito-borne virus (Gaunt *et al.*, 2001; Gould *et al.*, 2003; Calisher *et al.*, 1989). The most divergent of the mosquito-borne viruses form a clade typified by the yellow fever virus (YFV). These viruses are found in the old world and are largely transmitted by *Aedes* mosquitoes. A subsequent divergence gave rise to further clades containing viruses associated with *Aedes* mosquitoes including some causing hemorrhagic disease, exemplified by the dengue virus serological group, and another with *Culex* mosquitoes causing encephalitis, typified by

members of JEV serological group (Porterfield, 1975). A study on origin and distribution of JEV in Southeast Asia revealed that all 5 genotypes of JEV are found in Indonesia, New Guinea and Malaysia suggesting that JEV originated from its ancestral virus in the Indonesia-Malaysia region and evolved there into different genotypes which then spread across Asia (Solomon *et al.,* 2003). The phylogenic clustering and relationship have been in agreement with the classification of flavivirus using the standard classification scheme. The Japanese encephalitis serological group of flaviviruses includes 8 virus species and 2 subtype viruses with a geographical range encompassing all continents except Antarctica. The major virus species in this group and their geographic distribution is as follows:

1. JEV in Eastern, Southern and Southeastern Asia, Papua New Guinea and the Torres Strait of Northern Australia.
2. West Nile virus in Africa, Southern and Central Europe, India, The Middle East and North America and Kunjin virus (Subtype of West Nile virus) in Australia and Papua New Guinea.
3. The Murray Valley encephalitis virus (MVEV) in Australia, Papua New Guinea and the Western Indonesia archipelago.
4. St. Louis encephalitis virus (SLEV) in North and South America.
5. The other minor members in JE serology group are Usutu, Koutango and Yaounde viruses which are prevalent in Africa; Cacipacore virus in South America; and Alfuy, a subtype of Murray Valley Encephalitis virus, in Australia.

Most of these viruses have avian vertebrate hosts and are transmitted by *Culex* mosquitoes.

The most members of *Flavivirus* genus are arthropod borne or ARBO viruses, a term that describes their requirement for a blood sucking arthropod to complete their life cycle. The flaviviridae genus comprises more than 70 viruses, 40 of which are mosquito borne, 16 tick borne and 18 have no known vector (Heinz *et al.,* 2000).

2.5 Enzootic cycle of JEV

JE is a vector-borne viral disease. JEV is transmitted between vertebrate hosts by mosquitoes belonging to the *Culex* sp. The virus is able to replicate within the salivary glands

of the mosquitoes. Mature JE virions remain entrapped in intracellular vacuoles and are later released into the apical cavity of salivary gland cells through the fusion of these vacuoles with the apical plasma membrane. This process is associated with primary re-synthesis of saliva in mosquitoes following blood feeding activity. Another type of shedding involves virus particles, either singly or in mass, being released directly through the apical plasma membrane (Takahashi and Suzuki, 1979). Components of the mosquito saliva may also modulate infection by altering the local cytokine milieu. Feeding by mosquitoes of *Culex* sp. or administration of sialokinin-I, a mosquito salivary protein, have been found to downregulate IFN-γ production and upregulate the TH2 cytokines, IL-4 and IL-10 (Zeidner *et al.*, 1999). Human infections are mainly spread by *Culex tritaeniorrhynchus* which breeds in pools of stagnant water such as rice paddy fields (Innis *et al.*, 1995). Because the rice paddy is unavoidable, majority of the population in rural Asia has been infected with the virus by early adulthood (Solomon, 2003). Wading ardeid water birds (e.g. herons and pond egrets) and bats serve as virus reservoirs or maintenance hosts, but the virus regularly spills over into pigs, members of the family of Equidae (e.g. horses and donkeys) and humans. Pigs are considered as the main amplification hosts as viraemia results with a high titre. Due to the close proximity of pigs with human dwellings these animals are considered main component in the transmission cycle with respect to human infection (Ghosh and Basu, 2009). JEV infection in other domestic animals does not result in high viraemia and thus, they are not expected to transmit the virus to humans (Dutta *et al*., 2010).

2.6 Epidemiology

The geographic area where JE is endemic has increased over the last 70 years. The difference in diagnostic facilities and reporting of encephalitis in different regions renders the precise estimate difficult. The first epidemic of JE was reported in Japan in the 1930s. In China, outbreaks of summer encephalitis have been occurring since 1935. Currently there are 10,000–20,000 patients reported every year in China, although in the early 1970s the annual incidence was more than 80,000 patients (Vaughn and Hoke, 1992). In the far eastern Russian state, JE was first reported in 1938. In 1949, a large epidemic was reported in South Korea. In 1965, epidemics of JE were reported in Northern Vietnam with a current annual incidence of 1000–3000 cases. In 1969, JE epidemics occurred in Chiang Mai Valley in Northern Thailand and its current annual incidence is 1500–2500 cases. In India, JE was first reported

in Vellore, South India in 1955 and remained restricted until the 1970s (Webb and Pereira, 1956). Later JE epidemics spread to the whole of India including the costal areas, North India Sub Himalayan areas and Eastern India. Large annual outbreaks exceeding 2000–7000 cases are reported every year. In the late 1970s, JE cases were reported from Burma, Bangladesh and Southern Nepal. In Southern Nepal, about 500 cases of JE have been reported every year. In 1985, the first epidemic of JE was reported in Sri Lanka affecting 410 patients and 75 deaths. JE has also been reported from Pakistan.

In Malaysia JEV was isolated in the 1960s and about 100 cases are recorded annually. In Indonesia, JE is endemic and 1000–2500 cases occur annually; however in a majority of these patients, the etiological agent is not confirmed (Wuryadi and Suroso, 1989). In the Philippines and New Guinea sporadic JE cases have been reported. The first case of JE was reported from the Australian Torres Strait Island in 1995 and north of Cairns on the Australian mainland in 1998 (Hanna *et al.,* 1996; 1999).

Although JE is primarily concentrated in Southeast Asia, 3 epidemiological regions have been identified (Rhodin, 1996).

1. Endemic region: comprised of Southern India, Southern Vietnam, Southern Thailand, The Philippines, Malaysia and Indonesia. In this region, the mosquitoes are more often attracted by birds and pigs and human cases are rare.

2. Intermediary subtropical region: This includes Northern India, North and Central Burma, Northern Thailand, Northern Vietnam, Southern China and Bangladesh, JEV transmission is permanent but of low intensity; however, it increases to higher levels during rainy season especially April to October. Epidemics in contrast are severe and mostly affect children.

3. Temperate epidemic region: This region spans Northern China, Korea, Japan, Taiwan and Southern Russia. Transmission of JEV is variable depending on the environmental temperature. During winter, the mosquitoes are inactive but large epidemics may occur during summer and autumn. The geographic area of JE is increasing. The mechanism by which JE invades and establishes into new areas is not well understood. It has been thought for a long time that changing land usage and agriculture practices following

deforestation especially expansion of rice growing areas may be responsible for the increase in the incidence of JE. This however, may not be the only reason for invasion of JEV to new areas. Three possible mechanisms have been suggested.

1. Wind-blown mosquitoes
2. Bird migration
3. Movement and transportation of infected host.

Introduction of JEV into the Torres Straits was by migratory birds moving from Indonesia to New Guinea and finally to Torres Straits, where mosquito-pig and mosquito-bird cycles set in (Mackenzie *et al.*, 2002a, b). The subsequent movement of JEV from the Torres Straits into the Northern Mainland Australia was through the movement of infected mosquitoes blown by cyclonic winds (Ritchie and Rochester, 2001). The role of wind in spreading JEV is illustrated by annual blowing in of infected *Culex tritaeniorrhynchus* in China and Japan (Asahina and Noguchi, 1968).

In the developed countries such as Japan, Taiwan and South Korea, the number of JE cases has dropped considerably which may be due to mass vaccination of children, vector control, changing pig rearing practices, separation of housing from farming areas and better and clean ambience and reduced availability of mosquito breeding pools (Innis, 1995). In these areas control of JE in children and declining immunity in adults has led to reports of JE in the elderly (Vaughn and Hoke, 1992).

2.6.1 Epidemic cycle

JEV is transmitted through a zoonotic cycle between mosquitoes, pigs and water birds. Humans get accidentally infected when bitten by an infected mosquito and are a dead end host. Humans do not participate in the spread of JE because of low level and short-lived viremia (Misra and Kalita, 2010).

2.6.2 Vertical transmission of JEV

The vertical transmission of JEV refers to transmission of the virus to the next generation of mosquitoes. Vertical transmission probably occurs at oviposition rather than transovarially which might account for the persistence of virus in nature (Rosen *et al.*, 1989). Vertical

transmission of JEV has been reported in 3 strains of *C. tritaeniorrhynchus, C. pipiens, Aedes albopictus, A. togoi, C. annulus, C. quinquefasciatus* and *Armigeres subalbatus* mosquitoes.

2.6.3 Animal reservoirs

The antibody to the JEV in different animals provides an estimate of degree of exposure and susceptibility of respective species to JEV infection. A high prevalence of JEV antibodies has been documented in pigs, horses and birds and to a lesser extent in cattle, sheep, dogs and monkeys. Pigs and ardeid birds are the most important hosts for maintenance, amplification and spread of JEV. Pigs are the main component in the transmission cycle with respect to human infection whereas herons, egrets and other ardeid birds are important maintenance hosts. JEV infected animals and mosquitoes generally remain asymptomatic, although fatal encephalitis occurs in horses and fetal wastage occurs in swines (Burke and Leake, 1988). These effects on domestic animals have lead to the development of animal vaccines. The domestic animals can get infected but show no evidence of viremia. Rodents appear to be unimportant hosts (Scherer *et al.,* 1959b). Amphibians, reptiles and bats can become infected experimentally and the virus can persist. Pigs are the most important reservoir of JEV because of the following reasons:

(1) High incidence of natural swine infection.
(2) High frequency of viremia in pigs after infected mosquito bite.
(3) Viremia lasting in high titer for 2–4 days which is adequate to infect *C. tritaeniorrhynchus*.
(4) Transmission of JEV from pig to pig by laboratory reared *C. tritaeniorrhynchus*
(5) Large number of *C. tritaeniorrhynchus* mosquitoes found biting pigs in nature.
(6) Presence of large number of susceptible pigs is replenished each year due to commercial slaughtering of the animals at 10–16 months of age (Scherer *et al.*, 1959a, 1959b).

2.6.4 Bird mosquito cycle

Bird mosquito cycle is thought to be important in maintaining and amplifying JEV in the environment. Viremia frequently follows infection of both wild and domestic birds following the mosquito bite. The viral titers in the birds are adequate to infect other mosquitoes. In India,

34.8% of pond herons, egrets and cattle have JEV neutralizing antibodies (Rodrigues *et al.*, 1981). Japanese encephalitis virus antibody is passively transferred from immune hens as well as from 25% of immune wild herons and egrets to their progeny which are detectable until the third to fifth week of life (Buescher *et al.*, 1959b). Antibody-positive young birds, however, can be infected with an adequate dose of virus. Once the birds have been infected, they are immune and no longer able to amplify JEV (Scherer *et al.*, 1959c; Miyamoto and Nakamura, 1969).

2.6.5 Effect of seasons

In the tropics, JEV transmission occurs round the year whereas seasonal epidemics begin during the rainy seasons when the mosquito density is maximum (Umenai *et al.*, 1985). An epidemiological survey in South India (Tamilnadu) demonstrated a sequence of increasing rainfall followed by an increase in vectors, seroconversion in sentinel farm animals, followed by seroconversion and encephalitis in humans (Mani *et al.*, 1991). Similar phenomena have been observed in other countries as well. In Thailand, non immune sentinel pigs in the dry hot season do not get infected until several weeks after the first rains of the wet season occur (Hoke *et al.*, 1988). Two epidemics, during April to July (severe) and during September to December (milder), have been reported from Karnataka, India. Broadly speaking, two epidemiological patterns of JE are recognized in the northern areas which include North Vietnam, Northern Thailand, Korea, Japan, China, Taiwan, Nepal and Northern India in the Northern region large epidemics occur during the summer months. Whereas in southern regions such as South Vietnam, southern Thailand, Indonesia, Malaysia, The Philippines, Sri Lanka and Southern India, JE tends to be endemic and cases occur sporadically throughout the year with a peak during the rainy reason. This difference between southern and northern regions is more closely related to temperature rather than rainfall. In a study from Vietnam, the rainfall between north and south was similar. The temperature remained high throughout the year in the south as well as the number of JE cases. Whereas in the north, a sharp rise in JE cases occurs during the summer months which corresponds with the rise in temperature above 20^{o}C. At lower temperatures, the larval development time and extrinsic incubation period of JEV are longer which may also reduce the rate of virus transmission.

It is difficult to understand how the JE virus survives in the cold season in temperate and subtropical endemic areas. There are several hypotheses to explain this phenomenon. The virus may overwinter in hibernating mosquitoes, in mosquito eggs, in reptiles or it may be reintroduced by migrating birds. In Korea, a collection of 50,499 mosquitoes during the winter months over a 6 year period revealed 2 strains of JEV, one in the month of December and another in February (Lee, 1971). JEV has also been isolated from larvae collected in the month of June suggesting vertical transmission of JEV as a possible explanation for overwintering (Rosen *et al.*, 1989). Maintenance of JEV in mosquito may be the principal method of overwintering (Rosen, 1986). The migrating birds in southern areas may reintroduce JE in temperate zones during summer. It is, however, unlikely because the adult migrating birds are resistant to experimental infection by JEV (Buescher, 1956). The reactivation of latent JEV has been suggested due to stress of migration or hormonal changes. This phenomenon has however been ruled out by demonstrating different strains of JEV in northern and southern regions (Chen *et al.*, 1990). The role of snakes and frogs in overwintering of JEV has been suggested from Korea. Two JEV isolates were obtained from 747 snakes (Lee *et al.*, 1972). Under artificial hibernation conditions, the virus could be detected in snakes and frogs after 6 months (Oh *et al.*, 1974).

2.6.6 Clinical manifestations in humans

The incubation period in man, after a mosquito bite, is not exactly known. In general, it varies from 1–6 days. However, it can be as long as 14 days. The manifestations of the disease depend on which part of the nervous system is affected and include early symptoms, such as nonspecific febrile illness, headache, aseptic meningitis and encephalitis. *viz*. diarrhea and rigor, followed by symptoms such as reduced levels of consciousness, seizures, headache, photophobia, and vomiting in the next stage (Solomon *et al.*, 2000). In some cases, abnormal mental conditions may be observed. Later symptoms also include poliomyelitis-like flaccid paralysis (Solomon *et al.*, 1998) and parkinsonian syndrome, which manifests the classic description of JE-dull, flat, mask-like face with wide, unblinking eyes; tremor; generalized hypertonia, cogwheel rigidity and other abnormalities in movement (Solomon *et al.*, 2000). Severe encephalitis is associated with a higher frequency of seizures (Solomon, 2004). In fatal cases, patients ultimately slip into an acute coma. Various electroencephalographic abnormalities

include the presence of alpha, theta, and delta coma, and epileptiform conditions (Solomon *et al.*, 2000; Kalita and Misra, 1998; Misra and Kalita, 2010). Symptoms of brainstem infection include changes in the respiratory pattern, flexor and extensor posturing and abnormalities in the papillary and occulocephalic reflexes (Solomon *et al.*, 2000; Kumar *et al.*, 1990). In fatal cases of JEV, pathological changes are polymorphic and diffuse, involving various parts of the nervous system where the brain shows a severe degree of vascular congestion, microglial proliferation, formation of gliomesenchymal nodules, focal or confluent areas of cystic necrosis, cerebral edema, and transcompartmental shift (Gourie-Devi *et al*., 1995). Many survivors of JE acquire neuropsychiatric sequelae with cognitive and language impairment, in which case the disease presents itself not only as a killer but also as a cause of an immense social and financial burden, especially for a developing country (Kaur and Vrati, 2003; Vaughn and Hoke, 1992; Misra and Kalita, 2010).

2.6.7 JEV infection in Pigs

The primary disease manifestation of JEV infection in sows and gilts is reproductive failure manifested by abortion and abnormal farrowings. Litters contain stillborn and mummified fetuses, and weak neonates that may present with hydrocephalus and subcutaneous edema. Sexually mature swine do not show any significant clinical signs of infection, but transient anorexia and a mild febrile response have been observed. Reproductive failure occurs in non immune sows that become infected before 60-70 days of gestation. Infection after this time does not appear to affect piglets significantly (Sugimori *et al.*, 1974). JEV has also been associated with infertility in boars. Hashimura *et al*. (1976) isolated JEV from the testicles of a boar with orchitis. Ogasa *et al*. (1977) showed that infection of susceptible boars resulted in edematous, congested testicles that produced semen with numerous abnormal spermatozoa and significantly depressed total and motile sperm counts. Yamada *et al*. (2004) reported that JEV was isolated from tonsils of 2 of 4, 40 day old pigs that presented with a wasting syndrome. These pigs did not exhibit neurological signs, but histological examination revealed a nonsuppurative meningoencephalitis. These investigators were able to reproduce nonsuppurative meningoencephalitis in 3-week-old piglets with the isolated viruses. The affected pigs also showed varying degrees of depression and hind limb tremors, whether or not an association with a wasting syndrome exists was not established, but it is noteworthy that JEV infection in

humans can present with wasting of limbs as a result of flaccid paralysis (Solmon and Vaugh, 2002). Kodama *et al*. (1968) also reported that experimental infection of 2-day old piglets produced tremors and paralysis of the hind limbs**.**

2.7 Emerging JE

The emergence of JE can probably be explained by 2 factors. First, JE-endemic countries experienced an unprecedented population growth in recent decades. For example, in Eastern Asia, South-Central Asia, and Southeast Asia, the population more than doubled, from 1.7 billion in the mid-1950s to 3.5 billion 50 years later (United Nations, 2005). Second, pig rearing has grown exponentially and rice-production systems, particularly irrigated rice farming, have increased both in cropping area and cropping intensity. In China, for example, pork production doubled from 1990 to 2005.

Today, the total rice-harvested area of all JE-endemic countries (excluding the Russian Federation and Australia) is 1,345,000 km^2, an increase of 22% in the past 40 years. Over the same time span, the total rice production in these countries has risen from 226 million tons to 529 million tons (+134%). The number of people living in close proximity to irrigated areas reflects the fraction of the population that potentially is at an elevated risk of acquiring JE. The absolute and relative change of irrigated rice area and the relative change in pork production can be used as proxies for alterations in the risk of acquiring JE. In absolute numbers (116.6 million) and in relative terms, most people living in close proximity to irrigated areas are from Bangladesh (82%); the second largest population lives in India (107.8 million), followed by China (22.0 million). The largest irrigated rice area in 2005 was found in India (41.9 million ha), followed by China (29.0 million ha), Bangladesh (10.5 million ha), and Thailand (10.0 million ha). Highest increases in irrigated rice areas in the past 15 years were estimated for Myanmar (+47%) and Cambodia (+30%). Highest increases in pork production occurred in Myanmar (+381%), Vietnam (+147%), and China (+87%). On the other hand, pork production declined in Malaysia (–47%), North Korea (–35%), and Japan (–23%). Despite the fact that irrigated rice production and pig rearing are key factors in the transmission of JE, crude numbers fail to completely explain the complex interplay of various contextual determinants of the disease. Clearly, where rice production and pig rearing overlap, the impact on JE transmission is stronger than in areas where both activities are physically separated. This is the case, for example, in

Malaysia, where the Malays mainly grow rice in 1 area and the Chinese rear pigs in another area. Here, the social determinant of religion (most Malays are Muslim) plays a decisive factor (Erlanger *et al.*, 2009).

2.8 Global Prevalence/Incidence of JE

2.8.1 Human

The annual incidence of the JE is 30,000–50,000 cases globally out of which 10,000 to 15,000 deaths are occurring annually due to this disease (Solomon, 2006) and the estimated global impact from JE in 2002 was 709,000 disability-adjusted life years (DALYs) (Solomon, 2006). In the past 50 years the geographical area affected by Japanese encephalitis virus has expanded. Differences in diagnostic capabilities and in reporting of encephalitis make it impossible to plot this expansion precisely (Solomon *et al.*, 2000). However, the timing of the first reported cases or new epidemics in each area gives an impression of the relentless spread of Japanese encephalitis. In China outbreaks of summer encephalitis occurred from 1935, and the virus was first isolated there in 1940; there are currently 10–20 000 cases a year, although in the early 1970s it was over 80 000 cases annually (Vaughn and Hoke, 1992). In the far eastern Russian states, Japanese encephalitis first occurred in 1938. In 1949, large epidemics were reported from South Korea for the first time. Epidemics in northern Vietnam followed in 1965 (currently 1000–3000 cases nationally a year), and in Chiang Mai in northern Thailand in 1969 (currently 1500–2500 cases nationally a year). Japanese encephalitis was recognised in southern India from 1955, but was confined to the south until the 1970s. Since then, large outbreaks (2000–7000 cases a year) have been reported from eastern and northeastern states. The fact that adults and children were equally affected in these Indian states strongly supports the idea that the virus was introduced here for the first time. The late 1970s also saw the first cases in Burma and Bangladesh, and large epidemics (up to 500 cases a year) in southwestern Nepal. In 1985 Sri Lanka experienced its first epidemic with 410 cases and 75 deaths. Japanese encephalitis virus continues to spread westward with cases occurring in Pakistan (Igarashi *et al.*, 1994) and new epidemics in the Kathmandu valley of Nepal (Zimmerman *et al.*, 1997). JE has been recognized in 1978 from Nepal and since then 2000-3000 cases with 200-400 deaths per year were reported annually from 27 districts of the country (Pant *et al.*, 2006). In 1997, large epidemic of JE occurred in three western districts Bake, Bardia and Kilali with

2403 cases and 335 deaths in Nepal (Akiba *et al.*, 2001). The disease has occurred on the western Pacific islands with outbreaks in Guam in 1947 and Saipan in 1990 (Paul *et al.*, 1993). In Malaysia the disease is endemic; the virus was first isolated in the 1960s and about 100 cases are recorded annually. Japanese encephalitis is endemic in Indonesia, and 1000–2500 cases of encephalitis are reported annually, although in most the etiological agent is not confirmed (Wuryadi and Suroso, 1989). Further east, Japanese encephalitis occurs sporadically in the Philippines and New Guinea. The first cases occurred in the Australian Torres Straits islands in 1995, (Hanna *et al.*, 1996) and it was reported for the first time north of Cairns on the Australian mainland in 1998.

The reasons for the spread of Japanese encephalitis are incompletely understood, but probably include changing agricultural practices, such as increasing irrigation (which allows mosquito breeding), and animal husbandry (which provides host animals). In Indonesia, the lower prevalence of antibody to Japanese encephalitis virus in Borneo than neighboring Bali has been attributed to the lack of pigs in this predominantly Moslem culture (Wuryadi and Suroso, 1989). In developed countries such as Japan, Taiwan, and South Korea the number of cases has fallen, probably due to a combination of mass vaccination of children, spraying of pesticides, changing pig rearing practices, separation of housing from farming, better housing with air conditioning, and less availability of mosquito breeding pools (Innis, 1995). However, in Korea the widespread use of vaccine in children has been associated with a higher incidence of Japanese encephalitis in those over 15 years (Vaughn and Hoke, 1992).

2.8.2 Animals and Birds

JEV infects large number of animals such as horses, swine, cattle, duck, chicken and reptiles in nature. During the periods immediately preceding epidemics, the infection in swine reaches very high levels. Infection in swine is generally inapparent, except stillbirths and abortions in pregnant sows when infected and aspermia in boars whereas infected horses shows signs of encephalitis (Murphy *et al.*, 1999; Burke and Monath, 2001). In southern Thailand, 70% of pigs are infected (Burke *et al.*, 1985). High antibody titers have been observed in equines, bovines and various species of wild and domestic birds. In China, ducks have been found to have reactor rates of over 20%. In Japan, an examination of 1,339 sera from cattle of different areas of the country revealed reactor rates of 59.7% in the central part of the country and

56.8% in the south, whereas in the north the rate was only 2.1% (Sakai *et al.,* 1985). In Japan, large number of epidemics of JE in horses were recorded in 1947 and 1948 (Yamanaka *et al.,* 2006). JE causes major economic losses in Asian countries like Japan, Taiwan and Thailand where high rate of abortion and neonatal mortality reported in swine population. In endemic areas of Asia a morbidity rate was estimated at 44.8 per 10,000 equines. In 1948 epidemic, morbidity rate rose to 337.1 per 10,000 equines in Japan (Acha and Syfrez, 2003). Pant *et al*. (2006) reported seroprevalence of JE to the tune of 61% in pigs, 50% in horses and 7% in ducks using competitive ELISA in Nepal. A serological survey of pigs, horses and ducks revealed a total of 43.92% seropositivity to Japanese encephalitis virus antibodies (Pant *et al.,* 2006). Seroprevalence of 21.7% of JEV in swine was reported by Yang *et al*. (2006) by I-ELISA. JEV antibodies were detected in 68% wild boars by IgG ELISA (Hamano *et al.,* 2007). The presence of neutralizing and haemagglutination inhibition (HI) antibodies to JEV was also found in wild pigs of Singapore and wild pigs could serve as sentinel for transmission of JEV (See *et al.,* 2002). Chang (2002) reported seasonal prevalence of JEV antibodies in pigs and suggested that JEV propagates and remain active in winter as well as other seasons of the year. The incidence of JEV was 51.3% among domestic cattle in Korea based on the haemagglutination inhibition test and results indicate that swine are susceptible hosts of bovine arboviruses without showing clinical symptoms in a natural environment (Lim *et al.,* 2007). Horimoto *et al*. (1987) reported monthly distribution of HI antibodies to JEV in cattle. Birds and pigs are the only known major effective viremic amplifying host serving as a source for infection in vector mosquitoes (Scherer *et al.,* 1980). Antibodies against JE have also been detected in bats, reptiles, lizards and snakes (Shortridge *et al.,* 1977; Doi *et al.,* 1983). Lee *et al*. (1956) reported seroprevalence of 21.7% in goats, whereas Yang *et al*. (2007) reported a seroprevalence of 12.1% in goats in Korea. In a recent study, in Japan, Ohno *et al*. (2009) reported seroprevalence of 83% wild boars and 59% raccoons. Shimoda *et al*. (2010) reported seroprevalence of 17% in dogs and 1% cats in Japan.

2.8.3 Indian Scenario

2.8.3.1 Human

In India, JE was first recognized in 1955 from cases of encephalitis admitted to the Christian Medical College and Hospital, Vellore, Tamil Nadu (Webb and Pereira, 1956). In

1958, JEV was isolated from wild caught mosquitoes in the same area and isolated from brain tissue of human cases (Reuben and Gajanana, 1997). The disease was remained confined to southern India until 1970s. The first major outbreak of JE involving more than 700 cases and 300 deaths occurred in Burdwan and Bankura districts of West Bengal in 1973 (Chakravarthy *et al*., 1975). Since then, the number of outbreaks have been reported from the states of Bihar, Uttar Pradesh, Assam, Manipur, Andhra Pradesh, Karnataka, Madhya Pradesh, Maharastra, Tamil Nadu, Haryana, Kerala, West Bengal, Orissa and Union territories of Goa and Pondicherry (Park, 2005).

In Uttar Pradesh, JE was first reported in 1978 in Gorakhpur district (Mathur *et al*., 1982; Khare, 1984). The incidence of JE rose steadily in Gorakhpur district during 1982-1988. There were 118 cases with case fatality rate of 23.7% in 1982 and by 1988, the number reached to 772 cases and 32.2% case fatality rate was reported (Kar *et al.*, 1992). An epidemic of viral encephalitis was reported from July through November 2005 in Gorakhpur, Uttar Pradesh, India. It was the longest and most severe epidemic in 3 decades; 5,737 persons were affected in 7 districts of eastern Uttar Pradesh, and 1,344 persons died (Parida *et al*., 2006b). Several studies on seroprevalence have established the presence of JEV antibodies in human population from various states in India (Kar *et al.*, 1992; Vajpayee *et al.*, 1992; Kanojia *et al.*, 2003; Padbidri *et al.*, 2002; Rao *et al.*, 2000). The varying prevalence rates for JE ranging from 20 to 41% was recorded by various researchers in human (Kumar *et al.*, 2006; Kari *et al.*, 2006; Narasimham *et al.*, 1988; Kanojia *et al.*, 2003; Mathur *et al*., 1990 and Padbidri *et al*., 2002). Phukan *et al*. (2004) reported a seroprevalence of 53.7% during 2000-2002 in Assam. In Bihar, reported 360 cases with 64 deaths (CFR of 17.8%) in 2005. In Andhra Pradesh, reported 873 cases and 178 deaths in 15 districts during the year 1999 (Rao *et al.*, 2000). During the year 2003, JE cases were reported from Warangal and Karim Nagar districts of Andhra Pradesh (Das *et al*., 2004).

2.8.3.2 Animals and Birds

Japanese encephalitis is endemic in various states in India and virus isolation and seropositivity to JEV specific antibodies is common in animals and birds (Geeverghese *et al.*, 1987a,b; Angami *et al.*, 1989; Mall *et al.*, 1995; Sarkar *et al.*, 1995; Kumanan *et al*., 2002; Raut *et al*., 2003). Prevalence of antibodies to JE among wild birds of Andhra Pradesh was

studied by Rodrigues *et al*. (1981) revealed 34.8% neutralizing antibodies to JE in pond herons and cattle egrets. Their findings suggested the role of these ardeid birds in natural JE transmission cycle in India. Loach *et al*. (1983) reported JEV antibodies in ducks, fowls and peridomestic sparrows from Bihar. Geeverghese *et al*. (1987b) isolated JEV from sentinel pig in Kolar. Seroprevalence of JEV antibodies was reported to the tune of 77.7% dogs, 52% cattle, 34% pigs, 21% goats, 21.4% pigeons and 22.2% heron egret's in Nagaland (Angami *et al*., 1989). In Bareilly, Uttar Pradesh, the HI positivity was found in dogs (55.77%), pigs (40%), horses (37.65%), buffaloes (21.92%), goats (17.86%), sheep (2.38%) and cattle (1.98%) (Mall *et al*., 1995). Ratho *et al*. (1999) reported JE specific HAI antibodies in 30.3% pigs. Presence of HI antibodies to JE and West Nile viruses (WNV) has been recorded in the naturally infected pigs over a prolonged period (Geeverghese *et al*., 1994). Kumanan *et al*. (2002) reported highest incidence of JE in pigs (26.4%) followed by birds (9.37%), cattle (6.86%) and buffaloes (5.06). Recently, Nagaleelavathi *et al*. (2008) reported seroprevalence of 18% in pigs in Haryana. In a recent study, Kolhe, (2008) recorded IgG antibodies to JEV in 27.66% pigs and 8.07% birds and IgM antibodies in 28.89% pigs. In pigs higher prevalence was recorded in northeast region (35.22%), followed by Chandigarh (31.03%), Goa (29%), Deonar slaughter house Mumbai (25.89%) and Bareilly (20.83%).

2.9 Diagnosis

2.9.1 Aetiological diagnosis

Aetiological diagnosis of JE is based on virus isolation or demonstration of virus specific antigen or antibodies in CSF/blood. The laboratory diagnosis of a confirmed case of Japanese encephalitis is based on one of the following.

1. Fourfold or greater rise in serum antibody titer
2. Isolation of virus from or demonstration of viral antigen or genomic sequences in tissue, blood, CSF, or other body fluid
3. Specific IgM antibody by enzyme immunoassay (EIA) antibody captured in CSF or serum.

2.9.2 Culture

Isolation of JEV was conventionally carried out by intracerebral inoculation of clinical specimens in suckling mouse brain. Various cell cultures that are being used more recently include primary chick, duck embryo cells, and cell lines of Vero, C6/36, and AP61 cells. Virus can be isolated from the blood of patients in preneuroinvasive and neuroinvasive phases of the illness, usually not later than six or seven days after the onset of symptoms (Kim-Thoa *et al.,* 1974; Kedarnath *et al.,* 1984). However, isolation of virus from clinical specimens is generally considered a rare occurrence (Shope and Sather, 1979) probably because of low viral titres, rapid production of neutralising antibodies, and the logistic difficulty in transportation of specimens in developing countries and frequent freezing and thawing of clinical material (Mohanrao *et al.,* 1983; Leake *et al.,* 1986). Sensitive mosquito inoculation techniques have been described for isolation of JEV (Gajanana *et al.,* 1995). Identification of JEV in culture substrates was traditionally carried out by the complement fixation test and agar gel diffusion. The neutralisation test, monoclonal based immunofluorescence technique (IFT), and enzyme immunoassay (EIA) are presently being used (Zhang *et al.,* 1984).

2.9.3 Antigen detection

Various studies have proved the efficacy of antigen detection in CSF using reverse passive haemagglutination, (Ravi *et al.,* 1989) immunofluorescence, (Zhang *et al.,* 1989) and staphylococcal coagglutination tests using polyclonal or monoclonal antibodies (MAb) (Raghava and Badrinath, 1998) in rapid diagnosis of JE. Modified techniques such as use of M-IGSS have been successfully tried in the detection of antigen in mononuclear cells of peripheral blood (PBMC) and CSF of patients (Deng *et al.,* 1994).

2.9.4 Antibody detection

IgM antibody capture ELISA (Mac-ELISA) is the method of choice to demonstrate virus specific antibody in both blood and CSF (Bundo and Igarashi, 1985). However, when serum IgM antibodies are used for confirming JE, the co-presence of IgG antibodies should be demonstrated by another serological assay. Avidin biotin system (ABC Mac-ELISA), (Chow *et al.,* 1992) biotin labelled immunosorbent assay to sandwich ELISA, (Chang *et al.,* 1984a,b) nitrocellulose membrane based IgM capture dot enzyme immunoassay (Mac DOT),

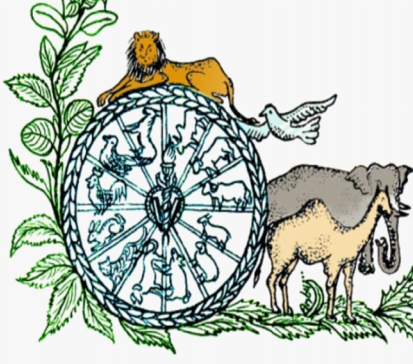
(Solomon *et al.,* 1998) and antibody capture radioimmunoassay (ACRIA) (Burke *et al.,* 1982) are some of the newer modifications of Mac-ELISA that have been used in antibody detection. Other serological tests such as haemagglutination inhibition, the complement fixation test, single radial haemolysis, and the neutralisation test are still in use in some laboratories. One of the earliest ELISA developed for the screening of swine sera was by Konishi and Yamaoka (1982). More recently an indirect ELISA (Yang *et al.,* 2006) and a quantitative ELISA (Xinglin *et al.,* 2005) were developed to detect antibodies against JEV in swine. Konishia and Kitai, (2009) developed an enzyme-linked immunosorbent assay (ELISA) to detect low levels of NS1 antibodies induced in humans with subclinical infections with JEV. At present, much advancement has been achieved in methods for the early detection of JEV; examples are the dipstick method (Shrivastva *et al.,* 2008) JEVCheX (Ravi *et al.,* 2006).

2.9.5 Molecular Diagnosis

Attempts to isolate Japanese encephalitis virus from clinical specimens are usually unsuccessful, probably because of low viral titers and the rapid production of neutralizing antibodies. However, all the above techniques are time consuming, labor intensive and are often cumbersome to adopt for routine clinical use. Although isolation of the virus from samples is essential to make a definitive diagnosis, RT-PCR has been used to detect *flavivirus* genome is a rapid and specific test (Eldadah *et al.,* 1991; Tanaka, 1993; Igarashi *et al.,* 1994; Meiyu *et al.,* 1997; Kuno, 1998; Paranjpe and Banerjee, 1998; Parida *et al*., 2006a; Sapkal *et al*., 2007; Saxena *et al*., 2009). Two-step RT-PCR assay requires agarose gel analysis for the detection of amplicons after PCR cycling. So, the assay is labor-intensive and has a very high risk of contamination. Recently, in addition to conventional RT-PCR, more rapid and sensitive real-time PCR-based assays, such as TaqMan RT-PCR, nucleic acid sequence-based amplification (NASBA), reverse transcription loop mediated isothermal amplification (RT-LAMP) and branched DNA methods, have been reported and are currently under extensive evaluation with human and field mosquito samples (Chan and Fox, 1999; Huang *et al.,* 2004; Igarashi, 1978; Parida *et al.,* 2006a; Pyke *et al.,* 2004; Shirato *et al.,* 2005). Chen *et al.* (2011) developed and evaluated RT-LAMP assay for detecting JEV. The sensitivity of the JEV RT-LAMP assay was in concordance with that of real-time RT-PCR and 10-times more sensitive than that of conventional RT-PCR.

The real-time PCR assay has many advantages over conventional RT-PCR methods, including rapidity, quantitative measurement, lower contamination rate, higher sensitivity, higher specificity, and easy standardization. Thus, nucleic acid based assays or real-time quantitative assay might eventually replace virus isolation and conventional RT-PCR as the new gold standard for the rapid diagnosis of virus infection in the acute-phase serum samples (Santhosh *et al.*, 2007).

Materials and Methods



3 MATERIALS AND METHODS

3.1 Biologicals and Chemicals

The biologicals and chemicals required in the present study were procured from the renowned International firms. The stock and working solutions used in the present study have been mentioned wherever necessary and listed in the appendix.

3.1.1 Reference Japanese encephalitis virus strain

In the present study, reference Japanese encephalitis virus (GP78) of human isolate, which was obtained from Department of Microbiology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow was used. This strain was maintained in Vero cell line in the Division of Veterinary Public Health, IVRI, Izatnagar and used throughout the study.

3.1.2 Enzymes

Taq DNA polymerase, Moloney murine leukemia virus (M-MuLV) reverse transcriptase, ribonuclease inhibitor, T4 DNA ligase and restriction enzymes were obtained from M/s Fermentas, Maryland, USA and used in the present study.

3.1.3 Vectors

CloneJET™ PCR cloning kit (Fermentas, USA) was used for cloning.

3.1.4 Prokaryotic host

E.coli Top10F' competent cells (M/s Invitrogen) used for cloning.

3.1.5 Real time-PCR kit

QuantiFast SYBR Green PCR Kit (400) (Quiagen) was used for detection and quantification of virus in pig blood samples.

3.1.6 Immunoconjugates

Goat anti-pig IgG heavy and light chain (Bethyl, Texas, USA) was used for indirect ELISA.

3.1.7 Chemical, glasswares, plasticwares and reagents

All the chemicals and reagents used in the present study were of analytical and molecular grades from Sigma, Hi-Media, SRL, BDH, Fermentas, Gentetix, Invitrogen, Genei Bengaluru, Hyclone and other reputed national and international firms. Similarly, glasswares and plasticwares were procured from Borosil, Qualigens, Axygen, Nunc and other firms of international repute. The plasticwares were either certified to be sterile, Nuclease and Nucleic acid free or were rendered RNAse and DNAse free by treating them with 0.1% DEPC overnight and autoclaved to inactivate DEPC.

3.1.8 Equipments, machines

Various equipments, instruments and machines used in the study are mentioned, wherever necessary.

3.1.9 Oligonucleotide primers

The Oligonucleotide primers used in the present study were got commercially synthesized from SBS Genetech Co., Ltd. and are listed in Table 3.1. The stock solutions of the primers were made in nuclease free water and stored at -20°C. The working solutions of the primers were made to a concentration of 10 pmol/µl and stored at -20°C.

Table 3.1: Oligonucleotide primers used in the study

Sl. No.	Primer name	Gene	Sequence (5'-3')	Primer length	Reference
Primers used in RT-PCR					
1	JE F	Envelope	CCATGGGTCTGGGAATGGGCAATCGTGAC	**29**	Self designed
2	JE R	(E)	CTCGAGTCACCCAACTTGCGCTGAATAAT	**29**	
Primers used in Real time PCR					
1	JERTF	NS3 (Non-structural	AGA GCG GGG AAA AAG GTC AT	**20**	Santhosh *et al.* (2007)
2	JERTR	protein 3)	TTT CAC GCT CTT TCT ACA GT	**20**	

3.2 Collection of samples

3.2.1 Materials required for Sample collection

10 ml syringes (18/20 gauge needles), vacutainers with anti-coagulant (Ethylene Di-amine Tetra-acetic Acid, EDTA) for whole blood and vacutainers with gel coated for serum, 70% ethanol, cotton and thermocol boxes with gel cool packs for transportation of samples at 4ºC.

3.2.2 Sample collection

A total of 854 samples (comprising 426 blood and 426 serum from 426 pigs) were collected from domestic pigs of (aged above 3 months) from different regions/areas considered endemic for Japanese encephalitis for the present study. The samples were collected during the period July 2011 to August 2012.

Table 3.2: Details of pig blood and serum samples collected during the study

Area/Farm from which samples were collected	Number of samples collected
Piggery farm IVRI, Izatnagar	30
Gorakhpur area	14
Balia district	72
Pig slaughter house, Bareilly	49
Deonar slaughter house, Mumbai	261
Total	**426**

3.2.3 Labelling and transportation of blood samples

Blood and serum samples were properly labelled and immediately transferred to the laboratory under chilled conditions and stored at -20°C till further processing.

3.2.4 RNA extraction from whole blood samples

The RNA was extracted with TRIzol-LS reagent (Invitrogen, Life Technologies, California, USA) as per the manufacturer's protocol. About 750 µl of TRIzol-LS reagent was mixed with 250 µl of blood sample and homogenized well. The homogenate was incubated for 5 min at room temperature to permit the lysis of sample. It was followed by addition of 0.2 ml

chloroform and vigorous shaking for 15 sec. The resulting mixture was incubated at room temperature for 10 min, followed by centrifugation at 12,000 g for 15 min at 4ºC. The colourless upper aqueous phase was transferred to fresh RNAase free eppendorf tube, to which 0.5 ml of 100% isopropanol was added for RNA precipitation. The mixture was incubated at room temperature for 1 h and centrifuged at 12,000 g for 10 min at 4ºC. The supernatant obtained was discarded and the RNA pellet was washed with 1 ml ethanol (75%) by centrifugation at 7500 g for 5 min at 4ºC. The ethanol wash was discarded and the RNA pellet was air dried for 5-10 min. The RNA pellet was resuspended in 25 µl of nuclease free water (NFW) or RNA storing solution (Ambion, Life Technologies, California, USA), followed by incubation in water bath set at 55-60ºC for 10-15 min and stored at -20ºC till further use.

3.2.5 Reverse Transcriptase-PCR (RT-PCR)

RT-PCR was standardized for detection of JEV using standard JEV RNA (GP78) extracted from infected Vero cell line. The RT-PCR was standardized for envelope 'E' gene using self designed primers (JEF and JER) listed in Table 3.1.

3.2.6 Extraction of ssRNA of JEV by Trizol method

The ssRNA of JEV was extracted with TRIzol-LS reagent (Invitrogen, Life Technologies, California, USA) as per the manufacturer's protocol. About 750 µl of TRIzol-LS reagent was mixed with 250 µl of cell culture suspension and homogenized well. The homogenate was incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complex. It was followed by addition of 0.2 ml chloroform and vigorous shaking for 15 sec. The resulting mixture was incubated at room temperature for 10 min, followed by centrifugation at 12,000 g for 15 min at 4ºC. The colourless upper aqueous phase was transferred to fresh RNAase free eppendorf tude, to which 0.5 ml of 100% isopropanol was added for RNA precipitation. The mixture was incubated at room temperature for 1 h and centrifuged at 12,000 g for 10 min at 4ºC. The supernatant obtained was discarded and the RNA pellet was washed with 1 ml ethanol (75%) by centrifugation at 7500 g for 5 min at 4ºC. The ethanol wash was discarded and the RNA pellet was air dried for 5-10 min. The RNA pellet was resuspended in 25 µl of nuclease free water (NFW) or RNA storing solution (Ambion, Life Technologies, California, USA), followed by incubation in water bath set at 55-60ºC for 10-15 min and stored at -20°C till further use.

3.2.7 Standardization of RT-PCR

Step 1: Synthesis of cDNA from ssRNA

The ssRNA was subjected to reverse transcription using specific primers targeting Envelope gene and M-MuLV reverse transcriptase enzyme. 0.2 ml PCR tubes were taken on ice and reaction mixture was prepared as follows, 2 µl (20 pmol) of reverse primer, 4 µl NFW and 5 µl of ssRNA. The tubes were spun and subjected to 65ºC for 10 min and 37ºC for 2 min in a Gradient palm-cycler™ (Corbett Research, Australia).

A master mix of 14 µl/reaction was prepared by adding 2.5 µl of 5x RT buffer, 1.0 µl of 0.1 M Dithiothreitol (DTT), 0.2 mM dNTPs, 0.5 µl RNAase inhibitor (40 U/µl), 0.5 µl of M-MuLV RT (20 U/µl) and 7 µl NFW and dispended in tubes containing RNA and primer.

After brief spin the tubes were loaded in palm-cycler with the following conditions.

1. 37°C for 60 min
2. 95°C for 10 min

Step 2: PCR amplification of cDNA

PCR was carried out in a final volume of 25 µl with following reagents with volume and cycling conditions for amplification of Envelope gene of Japanese encephalitis were depicted in table 3.2.

Table 3.3 : PCR reaction mixture and cycling conditions for amplification of Envelope gene

Reagents	Volume	Cycling conditions
10x PCR buffer (without $MgCl_2$)	2.5 µl	
MgCl2 (25 mM)	2.0 µl	Initial denaturation: 94ºC for 5 min
dNTP (2 mM)	2.5 µl	Denaturation: 94ºC for 45 sec } 40 cycles
Forward primer (10 pmol/µl)	2.0 µl	Annealing: 54ºC for 45 sec } 40 cycles
Reverse primer (10 pmol/µl)	2.0 µl	Extension: 72ºC for 45 sec } 40 cycles
Taq DNA polymerase (5 U/µl)	0.3 µl	Final extension: 72ºC for 5 min
cDNA	5.0 µl	
NFW	8.7 µl	Hold at 4ºC for 5 min
Total reaction volume	25 µl	

The PCR products were stored at -20°C until further analysis by agarose gel electrophoresis.

3.2.8 Agarose gel electrophoresis

Agarose gel electrophoresis was used to visualise the desired product obtained by the amplification of the ssRNA by RT-PCR. Agarose gel in the concentration of 1.5% was prepared by mixing Ultrapure™ Agarose (Invitrogen) in 1x TAE buffer (Appendix) and heated to dissolve it completely. The molten agarose was cooled to 50°C and ethidium bromide added (Geni, Bangalore) to a final concentration of 0.5 µg/ml before pouring in an evenly levelled gel tray already fitted with gel comb having its teeth 1 mm above the base and open ends sealed with adhesive tape. The gel tray was left undisturbed for about 30-40 min to allow the gel solidification, after which the comb was taken out and adhesive tape removed. The gel tray was then placed in submersive electrophoresis tank (Geni, Bangalore) with the wells side towards the cathode end and sufficient quantity of electrophoresis buffer (1x TAE), at least 1 mm above the gel surface.

The PCR product (10 µl) was mixed with 6x loading dye (2 µl) (Fermentas, USA) and loaded into a well along with a 100 bp DNA marker (Fermentas, USA) in the adjoining lane. The electrophoresis was performed at 75 V for 45 min and the mobility monitored by the movement of the dye. After sufficient migration, the bands (products) of expected size were observed and photographed using the gel documentation system (UV Products, Jena, Germany).

3.2.9 Detection of JEV in pig blood samples by RT-PCR

The standardized RT-PCR was used for detection of JEV in pig blood samples as per the protocol given above.

3.3 qRT-PCR for detection and quantification of JEV

The detection as well as quantification of JEV load in the pig blood samples was carried out by QuantiFast SYBR Green PCR kit (Quiagen) by following the protocol of Santhosh *et al.* (2007) and targeting the NS3 gene with necessary modifications.

3.3.1 Preparation of standard plasmid DNA

The standard plasmid was constructed by inserting the PCR fragment (162 bp) into a Pjet1.2/blunt cloning vector of CloneJET™ PCR Cloning kit (Fermentas) according to manufacturer's instructions. The RT-PCR was put for the known positive reference JEV available in the Division of VPH, IVRI, Izatnagar. In order to obtain purified PCR amplicons for cloning, the following approaches were followed.

3.3.2 PCR amplification of NS3 gene of JEV

3.3.3 Standardization of RT-PCR for NS3 gene

Step 1: Synthesis of cDNA from ssRNA

The ssRNA was subjected to reverse transcription using specific primers targeting NS3 gene and M-MuLV reverse transcriptase enzyme. 0.2 ml PCR tubes were taken on ice and reaction mixture was prepared as follows, 2 µl (20 pmol) of reverse primer (JERTR), 4 µl NFW and 5 µl of ssRNA. The tubes were spun and subjected to 65ºC for 10 min and 37ºC for 2 min in a Gradient palm-cycler™ (Corbett Research, Australia). A master mix of 14 µl/ reaction was prepared by adding 2.5 µl of 5x RT buffer, 1.0 µl of 0.1 M Dithiothreitol (DTT), 0.2 Mm dNTPs, 0.5 µl RNAase inhibitor (40U/µl), 0.5 µl of M-MuLV RT (20 U/µl) and 7 µl NFW and dispended in tubes containing RNA and primer.

After brief spin the tubes were loaded in palm-cycler with the following conditions.

1. 37°C for 60 min
2. 95°C for 10 min

Step 2: PCR amplification of cDNA

PCR was carried out in a final volume of 25 µl with following reagents with volume and cycling conditions for amplification of NS3 gene of Japanese encephalitis virus were depicted in table 3.3.

Table 3.4: PCR reaction mixture and cycling conditions for amplification of NS3 gene

Reagents	Volume	Cycling conditions
10x PCR buffer	2.5 µl	Initial denaturation: 94ºC for 5 min
MgCl2 (25 mM)	2.0 µl	Denaturation: 94ºC for 45 sec
dNTP (2 mM)	2.5 µl	Annealing: 54.5ºC for 45 sec } 40 cycles
Forward primer(JERTF) (10 pmol)	2.0 µl	Extension: 72ºC for 45 sec
Reverse primer (JERTR) (10 pmol)	2.0 µl	Final extension: 72ºC for 5 min
Taq DNA polymerase (5 U/µl)	0.3 µl	Hold at 4ºC for 5 min
cDNA	5.0 µl	
NFW	8.7 µl	
Total reaction volume	25 µl	

The PCR products were stored at -20°C until further analysis by agarose gel electrophoresis.

3.3.4 Agarose gel electrophoresis

Agarose gel electrophoresis was used to visualise the desired product obtained by the amplification of the ssRNA by RT-PCR. Agarose gel in the concentration of 1.5% was prepared by mixing Ultrapure™ Agarose (Invitrogen) in 1x TAE buffer (Appendix) and heated to dissolve it completely. The molten agarose was cooled to 50°C and added with ethidium bromide (Geni, Bangalore) to a final concentration of 0.5µg/ml before pouring in an evenly levelled gel tray already fitted with gel comb having its teeth 1 mm above the base and open ends are sealed with adhesive tape. The gel tray was left undisturbed for about 30-40 min to allow the gel solidification, after which the comb was taken out and adhesive tape removed. The gel tray was then placed in submersive electrophoresis tank (Geni, Bangalore) with the wells side towards the cathode end and sufficient quantity of electrophoresis buffer (1x TAE), at least 1 mm above the gel surface.

The PCR product (10 µl) was mixed with 6x loading dye (2 µl) and loaded into a well along with a 100 bp DNA marker (Fermentas, USA) in the adjoining lane. The electrophoresis was performed at 75 V for 45 min and the mobility monitored by the movement of the dye. After sufficient migration, the bands (products) of expected size were observed and photographed using the gel documentation system (UV Products, Jena, Germany).

3.3.5 Elution of PCR amplicons from agarose gel

Gel extraction of the PCR product was done using GeneJET gel extraction kit (Fermentas, USA) as per the manufacturer's instructions;

1. The amplified PCR products were run on 1% agarose gel and the gel slice containing the DNA fragment excised using a clean scalpel.
2. The gel was placed in a clean microcentrifuge tube and weighed.
3. Binding buffer 1:1 (w/v) volume was added to the gel slice and incubated in water bath at 50-60°C for 10 min or until the gel slice completely dissolved.
4. A volume of 800 µl of the solubalized gel solution was transferred to the GeneJET purification column and centrifuged at 10,000 rpm for 1 min. The flow was discarded and column placed back into the same collection tubes.
5. Wash buffer (diluted with ethanol) 700 µl was added to the GeneJET purification column and centrifuged for 1 min. The flow through was discarded and column placed back into the same collection tube.
6. The empty GeneJET purification column was then centrifuged for an additional 1 min to completely remove the residual wash buffer.
7. The GeneJET purification column was then transferred to a clean 1.5 ml microcentrifuge tube and 50 µl of elution buffer was added to the center of the purification column membrane.
8. The column was centrifuged for 1 min and the purified DNA stored at -20°C.

3.3.6 Ligation of PCR product into plasmid vector

The PCR products were ligated with desired vector as per the following reaction;

Table 3.5: Ligation reaction of PCR product with cloning vector

Component	Volume
2 x Reaction buffer	10 µl
PCR product	2 µl
Pjet1.2/blunt cloning vector (50 ng/ µl)	1 µl
NFW	6 µl
T4 DNA Ligase	1 µl
Total reaction volume	20 µl

The total volume was made upto 20 µl by NFW and vortexed briefly and centrifuged for 3-5 sec. Incubate the ligation mixture at room temperature 22°C for 5 min.

3.3.7 Preparation of *E.coli* Top10 competent cells

1. The *E.coli* Top10 cells, from frozen stock, were inoculated into 5 ml of LB broth without ampicillin and incubated overnight at 37°C in shaker incubator at 180 rpm.
2. The overnight grown culture ($1/10^{th}$) was inoculated into 5ml of LB broth and incubated for 2-4 h at 37°C in shaker incubator at 180 rpm.
3. About 1.5 ml of the culture was transferred to sterile microcentrifuge tubes and centrifuged at 12,000 rpm for 10 min at 4°C.
4. The supernatant was discarded, the microcentrifuge tubes kept on ice and 1 ml of 0.1 M $CaCl_2$ added with prechilled microtips. The pellet was gently resuspended in $CaCl_2$ and centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and 100 µl of 0.1 M $CaCl_2$ added with prechilled microtips. The tubes were then incubated on ice for 1 h before transformation.

3.3.8 Transformation of competent cells

5 µl of ligated product was added to competent cells and incubated on ice for 1 h followed by heat shock at 42°C for 90 sec and again incubation on ice for 10 min. The cells were then plated on LB plates containing ampicillin @ 100 µg/ml and incubated overnight at 37°C. The colonies obtained were screened for the presence of recombinant plasmid.

3.3.9 Screening of recombinant clones

The colonies obtained on the LB ampicillin plates were screened for presence of insert by the following methods;

1. Amplification of insert by colony PCR
2. Release of inset by RE digestion of the recombinant plasmid

3.4 Amplification of insert by colony PCR

Extraction of DNA by snap chill method

Two to three colonies obtained on LB plates after overnight incubation were inoculated in 5 ml of LB broth containing ampicillin (100 µg/ml) and incubated at 37°C for 4 h. Nearly

500 µl of the culture was taken in 2 ml centrifuge tubes and centrifuged at 10,000 rpm for 5 min. The supernatant was discarded and cell pellet was then dissolved in 25 µl of NFW and boiled for 10 min. It was then kept on ice for 5 min and centrifuged at 10,000 rpm for 5 min. The suspension was then used as template for setting up the PCR reaction as shown below in Table 3.4.

3.4.1 Colony PCR for screening of recombinant plasmid

Table 3.6: PCR reaction mixture for amplification of insert (NS3)

Component	Volume
10 x PCR buffer	2.5 µl
$MgCl_2$ (25 mM)	1.5 µl
Forward primer (10 pmol/µl)	1.0 µl
Reverse primer (10 pmol/µl)	1.0 µl
dNTPs (2 mM)	2.5 µl
Template DNA	2.0 µl
NFW	14.5 µl
Total volume	**25.0 µl**

The reaction mix was set up in a thermocycler as per the conditions described previously in Table 3.4. The amplified product was run on 1% agarose and visualised using the Gel documentation system.

3.4.2 Restriction enzyme digestion for release of insert

3.4.2.1 Extraction of vector plasmid DNA by alkaline lysis method

The plasmid DNA isolation was done by conventional alkaline method as per Sambrook and Russell (2001). Overnight grown culture (5 ml) was transferred to microcentrifuge tube and and centrifuged at 10,000 rpm for 2 min. The supernatant was thoroughly decanted, added 200 µl of resuspension buffer (PI) containing 100 µg/ml RNAse A and pellet was resuspended by vortexing the centrifuge tube. A volume of 200 µl of lysis buffer (PII) was added to the tube and mixed well. A volume of 200 µl of ice cold neutralization buffer (PIII) was added and mixed by inverting the tube for 5-6 times and incubated over ice for 20 min. The contents were centrifuged at 13,000 rpm for 20 min to pellet the precipitated DNA,

protein and RNA. The supernatant from the above step was transferred to a fresh microcentrifuge tube. To the aqueous phase, equal volume of isopropanol was added and kept for 20 min at room temperature. Plasmid pellet was obtained by centrifuging the tube contents at 13,000 rpm for 20 min. After decanting carefully decanting ethanol and dissolved in NFW. Plasmid was visualized on 1% agarose gel and stored at -20°C till further use.

3.4.2.3 Restriction enzyme digestion for release of insert

Restriction enzyme digestion was set up using *Xho*I and *Nco*I and the reaction mix (as per Table) was incubated at 37°C for 3 h. The released insert of the desired size was visualised by running on 1% agarose gel.

Table 3.7: RE digestion for insert release from recombinant plasmids

Component	Volume
10 x Tango buffer	3 µl
*Xho*I (10 U/ µl)	1 µl
*Nco*I (10U/ µl)	1 µl
Plasmid	10 µl
Total volume	**15 µl**

3.4.5 Standardization of Real time PCR with SYBR Green mastermix

The Quantitative real time RT-PCR was carried out by standard curve method using QuantiFast SYBR Green PCR Kit (400) (Quiagen). Reaction was performed in Mx3000P Real-time PCR system (Stratagene, USA) operated with MxPro™ QPCR software.

Synthesis of cDNA from ssRNA of reference JEV

The ssRNA was subjected to reverse transcription using specific primers targeting NS3 gene and M-MuLV reverse transcriptase enzyme. 0.2 ml PCR tubes were taken on ice and reaction mixture was prepared as follows, 2 µl (20 pmol) of reverse primer (JERTR), 4 µl NFW and 5 µl of ssRNA were added. The tubes were spun and subjected to 65ºC for 10 min and 37ºC for 2 min in a themocycler (Corbett, Australia).

A master mix of 14 µl/reaction was prepared by adding 2.5 µl of 5x RT buffer, 1.0 µl of 0.1 M Dithiothreitol (DTT), 0.2 Mm dNTPs, 0.5 µl RNAase inhibitor (40U/µl), 0.5 µl of M-MuLV RT (20 U/µl) and 7 µl NFW and dispended in tubes containing RNA and primer.

After brief spin the tubes were loaded in thermocycler with the following conditions:

1. 37°C for 60 min
2. 95°C for 10 min

The real time PCR reaction was run in duplicates, each in a total volume of 25 µl, comprising of the following components:

Table 3.8: Real-time PCR reaction mixture for amplification of NS3 gene

	Component	**Volume**
1)	2x QuantiFast SYBR Green PCR master mix	12.5 µl
2)	Forward primer JERTF (5 pM/µl)	0.6 µl
3)	Reverse primer JERTR (5 pM/µl)	0.6 µl
4)	Nuclease free water	10.3 µl
5)	c-DNA	1 µl
	Total volume	**25 µl**

Nuclease-free filter tips were used for taking the individual reaction components and preparation of reaction mixture. Careful pipetting was done without creating bubbles to avoid interference in reading of fluorescence by the instrument. After adding the above components, the strips were briefly spun and loaded on to the thermocycler.

The thermal profile for JEV comprised of three segments. Segment-1 comprised of initial denaturation at 95°C for 5 min followed by Segment-2 consisting of 40 repetitive cycles of denaturation at 95°C for 10 sec, annealing at 55°C for 15 sec and extension at 72°C for 15 sec each. Dissociation curve analysis was performed in segment-3 which comprised of a single cycle of denaturation at 95°C for 1 minute, partial renaturation to 55°C for 15 sec and denaturation again at 95°C for 30 sec. For generation of amplification plots, fluorescence

value (dR) was collected at second plateau (annealing phase) and to confirm fidelity of amplification, dissociation curve analysis was performed at the end of the run. After the Real-time PCR was completed, the amplicons of each sample were run in 2% agarose gel and visualized on a UV transilluminator.

3.4.6 Determination of initial copy number

In order to get PCR efficiency and slope value, a standard curve method was employed using SYBR Green chemistry. The concentration of the standard linearized plasmid containing the 162 bp insert of NS3 gene of JEV was measured by Nanodrop (USA) and converted to genome copy numbers by using the molecular weight of DNA to construct standard curve. The concentration of the standard plasmid was measured as 3044 ng/µl. The genome copy number per micolitre was calculated using the formula,

$$\text{Copy number} = \frac{\text{Mass of DNA in grams/µl X Avogadro's number}}{\text{Template length X Average molecular weight of a base}}$$

Avogadro's number = 6.023×10^{23}

Average molecular weight of a base = 660 g/ml

Mass of DNA = 3044×10^{-9} g

Template length = 3136 bp

Thus, by using the above equation the initial copy number was determined as $8.858X10^{11}$ copies per microlitre. The plasmid DNA copies (10^9) worked out in the known concentration of standard plasmid (3044 ng/µl) were then serially diluted tenfold to get copy numbers ranging from 10^9 to 10^1. The standard curve in terms of regression line equation was drawn by plotting the known log copy number in the dilutions against the threshold values (Ct). The PCR efficiency and slope were determined as per the default setting of the instrument.

3.4.7 Detection and quantification of JEV in blood samples of pigs

The standardized qRT-PCR was used for detection of as well as quantification of JE virus in pig blood samples. The genome copy numbers per micoliter of the samples were estimated based on the regression line equation of standard curve, by using the following formula;

Log10 copy number = Ct (Threshold cycle) – y intercept/slope (Adams, 2006)

3.5 Indirect-Enzyme linked immunosorbent assay (I-ELISA)

Whole viral antigen based indirect ELISA which was developed and standardized by Rawat (2013) was used in the present study for screening of JE in pig serum samples. Briefly, ELISA plates (Maxisorp, Nunc, Denmark) were coated with 50 µl/well of coating buffer (pH 9.6), containing JEV antigen (4 µg/ml) and incubated at 4°C overnight. The coated plates were washed three times with PBS-T (0.05% Tween 20) manually to remove excess unabsorbed antigen. The unblocked sites were blocked with 250 µl of 5% skimmed milk powder (SMP) (Gibco) in PBST and incubated for 2 h at 37°C. After incubation, plates were washed three times and 50 µl of (1:400) of JEV positive and negative sera in blocking buffer were added to the plate. It was incubated at 37°C for 1 h and then plate was washed three times with PBST. Goat anti-pig horse radish peroxidise (HRPO) conjugate was diluted 1:10,000 in blocking buffer and 50 µl added to each well, followed by incubation for 1 h at 37°C. The plate was washed three times again and freshly prepared substrate solution (10 ml citrate buffer, 10 mg OPD, 8 µl H_2O_2, pH 4.5) 100 µl added to each well in dark. Colour was allowed to develop for 5-15 min and reaction stopped with 2 N H_2SO_4 (50 µl). The absorbance was measured at 492 nm using ELISA reader (Microscan MS5605A, Electronics Corporation of India Ltd.). Serum samples showing a P/N ratio of $\geq$ 2 were considered positive for the standardized ELISA. The antigen concentration was standardized at 1:100. The primary and secondary antibody dilutions were standardized at 1:400 and 1:10,000, respectively.

3.6 Virus isolation

Attempts were made to isolate the JEV from RT-PCR positive blood samples of pigs as per standard methods of OIE (2004) and Gould and Clegg (1991). The virus isolation was carried out using monolayers of Vero cell line. The aliquots of 100 µl of filtered (0.22 µ) blood samples mixed with 400 µl minimum essential medium containing 2% fetal bovine serum and antibiotics (100 µg/ml streptomycin and 100 U/ml penicillin) and this mixture was used to inoculate the Vero cell line. The flasks were incubated in Co_2 incubator at 37°C for 1 h with slight rotating the flasks every 10 min of incubation. After 1 h of incubation the inoculums was decanted carefully and 5 ml of fresh maintenance medium was added to the flasks and were incubated at 37°C for 3-4 days.

The flasks were observed daily under inverted microscope after 48 h post infection for cytopathic effect (CPE). After three subsequent passages in cell line, the monolayer was observed for any cytopathic effect.

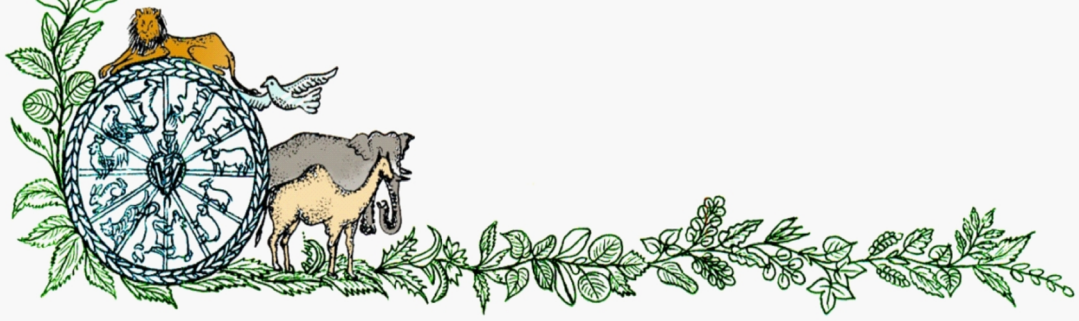
3.7 Cloning and sequencing of JEV positive amplicons

cDNA of two real-time PCR positive samples were amplified using primers targeting NS3 gene, purified and cloned using PGEMT vector as per the protocols mentioned earlier. The positive clones were sequenced by outsourcing it to M/s. Chromous Biotech Pvt.Ltd., Bangalore.

3.7.1 Sequence Analysis

The consensus sequences were prepared using SeqBuilder (DNASTAR, Lasergene). The pair wise nucleotide sequence homology was determined with previously published sequences of different JEV collected from NCBI and per cent identity was determined by CLUSTAL W of MegAlign (Lasergene DNAStar) software. Based on the deduced nucleotide sequences, phylogenetic dendrograms for GKP1_India_IVRI_VPH and GKP2_India_IVRI_VPH were analyzed with other previously reported sequences from different countries.

Results



4 RESULTS

In the present study, a total 852 samples (comprising 426 blood and 426 serum from 426 pigs) were collected from domestic pigs from various regions of Uttar Pradesh (Bareilly, Gorakhpur, Ballia) and Deonar Slaughter House, Mumbai, Maharashtra.

4.1 Prevalence of JEV by RT-PCR

4.1.1 Standardization of RT-PCR

RT-PCR was standardized for detection of JEV using standard JEV RNA extracted from GP78 infected Vero cell line. The RT-PCR was standardized for envelope 'E' gene using self designed primers and this gene segment yielded expected product size of 474 bp (Fig. 4.1).

Out of 426 pig blood samples, 17 (3.99%) were positive for JEV RNA, as the cDNA from these samples when amplified by PCR revealed the expected product of 474 bp (Fig. 4.2 & 4.3).

With the available data when analyzed further indicated that, samples from Uttar Pradesh only showed positivity by RT-PCR i.e., out of 165 pig blood samples 17 (10.30%) were positive for JEV RNA. The results of RT-PCR are presented in table 4.2.

4.2 Standardization of RT-PCR for NS3 gene

RT-PCR was standardized for detection of JEV using standard JEV RNA extracted from infected Vero cell line. The RT-PCR was standardized for NS3 gene using primers (JERTF and JERTR) and this gene segment yielded expected product size of 162 bp (Fig. 4.4).

4.2.1 Detection and quantification of JEV by Real time PCR

The detection as well as quantification of Japanese encephalitis viral load in the pig blood samples was carried out by Quantitative Real-time PCR assay by using the primers against the NS3 gene of JEV, with amplification product of 162 bp in length.

4.2.2 Preparation of standard plasmid DNA

The purified PCR product of 162 bp was successfully cloned in Pjet1.2/blunt cloning vector for the construction of standard plasmid DNA and confirmed by the release of insert from the plasmids after digestion with *Xho*1 and *Nco*1 restriction enzymes (Fig. 4.5).

4.2.3 Standardization of the assay

The standardized qRT-PCR assay for JEV with employing primers against the NS3 gene allowed the amplification of a 162 bp product. The absence of any primer-dimer in the agaorse gel indicated that the fluorescence from intercalated SYBR Green dye was obtained from the specific amplified product. The amplification plots and dissociation curves from the plasmid DNA served as a base for detecting JEV and its quantification (copy number) in the pig blood samples. To check the assay specificity, melting point analysis was performed. The Tm value was recorded as 83.7°C after completion of the dissociation (melting) curve (Fig. 4.10).

4.2.4 Interpretation of copy number

To quantify the copy numbers contained in the samples, a standard curve was prepared with 10 fold serial dilutions of plasmid DNA (concentration, 3044 ng/µl) containing the target gene. On plotting the known plasmid DNA copy numbers in serial tenfold dilutions (10^{10} to 10^{2} copies) against the corresponding average 'Ct' (threshold cycle) values i.e., 8.8, 11.96, 15.39, 19.25, 21.79, 25.55, 28.78, 31.26 and 33.29 respectively, a standard curve was obtained that served as a basis for estimating the number of genome copies of JEV in the positive blood samples of pigs (Fig. 4.6, 4.7, 4.8, 4.9 & 4.10). Linear regression of the 'Ct' values and the quantity of plasmid DNA revealed a good linearity (r^2 = 0.995, slope = -3.149 and efficiency=107.8%).

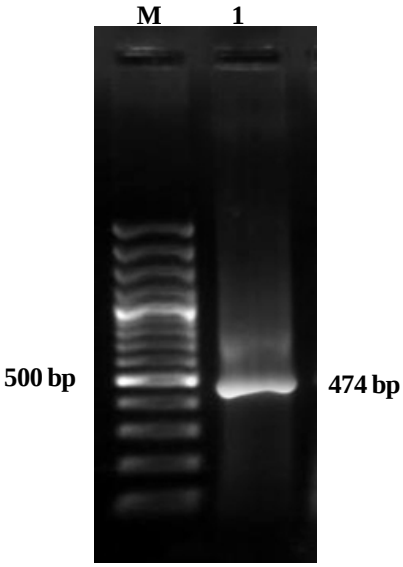


Fig. 4.1: Standardization of RT-PCR using JEV strain GP-78
Lane M : 100 bp DNA ladder
Lane 1 : Positive control

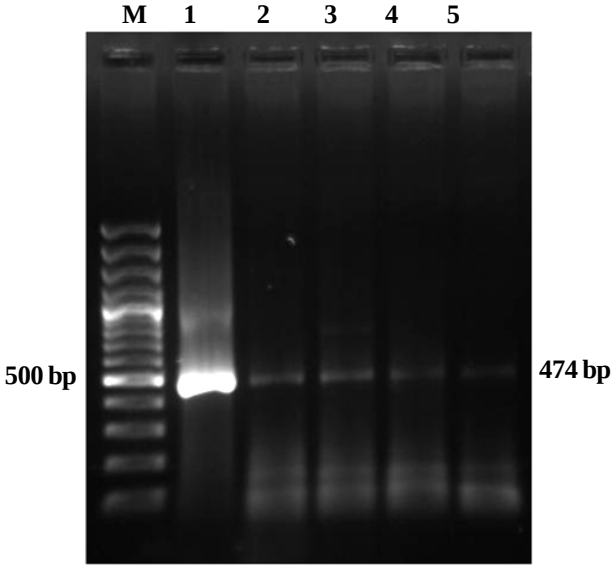


Fig. 4.2: RT-PCR based detection of JEV in Pig blood samples
Lane M : 100 bp DNA ladder
Lane 1 : Positive control
Lane 2-5 : JEV Positve samples

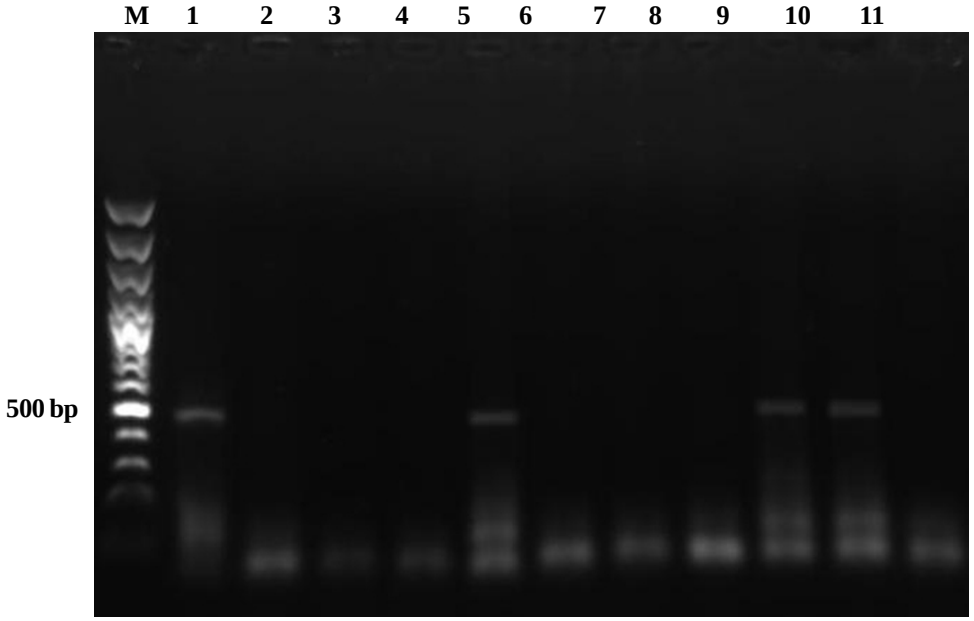


Fig. 4.3 : RT-PCR based detection of JEV in Pig blood samples

Lane M : 100 bp DNA ladder
Lane 1 : Positive control
Lane 5, 9-10 : JEV Positve samples

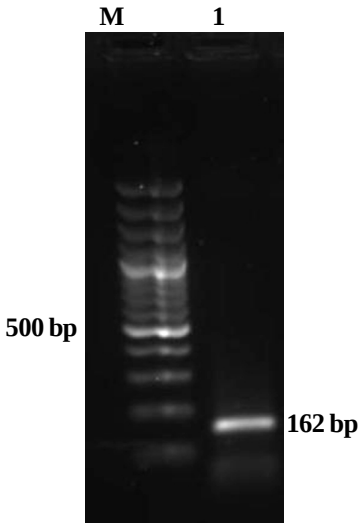


Fig. 4.4 : Standardization of RT-PCR for detection of JEV by targeting NS3 gene
Lane M : 100 bp DNA ladder
Lane 1 : Positive control (162 bp)

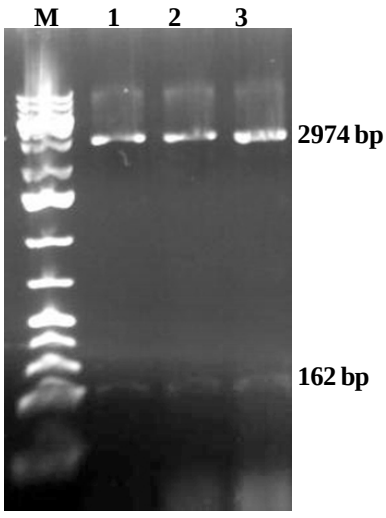


Fig. 4.5 : Release of insert from CloneJET™ vector after RE digestion
Lane M : 1 kb plus DNA ladder
Lane 1-3 : Plasmid with insert (162 bp)

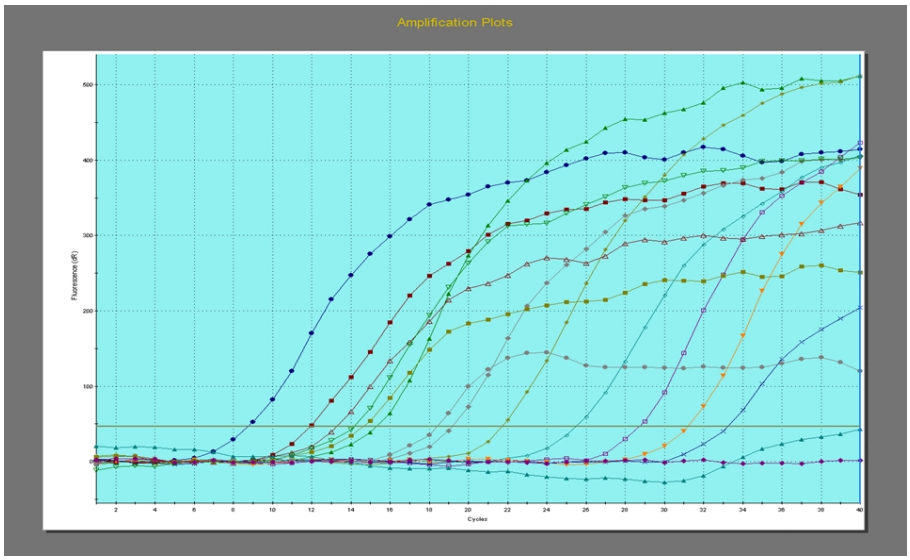


Fig. 4.6: Detection as well as quantification of JEV in pig blood samples based on the amplification plots generated by qRT-PCR

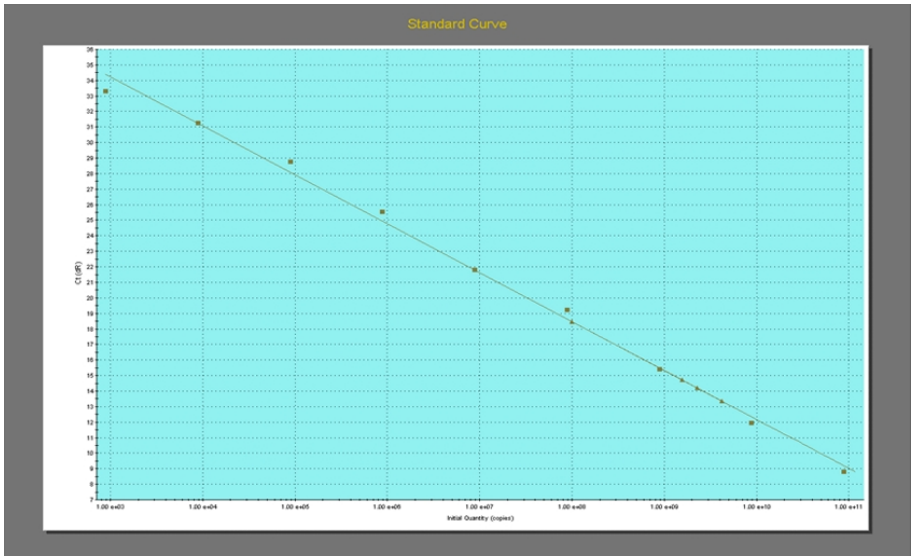


Fig. 4.7: Standard curve in qRT-PCR generated by plotting ten fold dilutions of standard plasmid DNA against the corresponding 'Ct' values

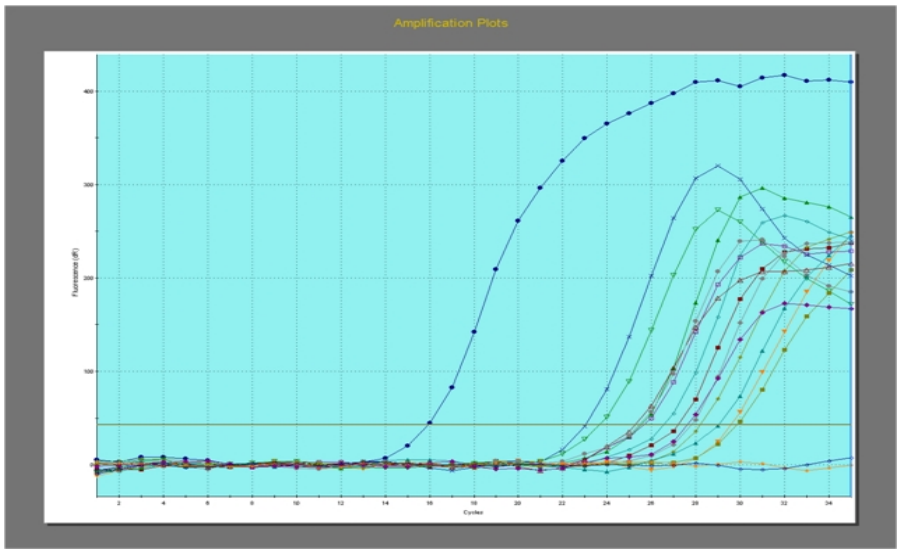


Fig. 4.8 : Amplification plots generated by qRT-PCR

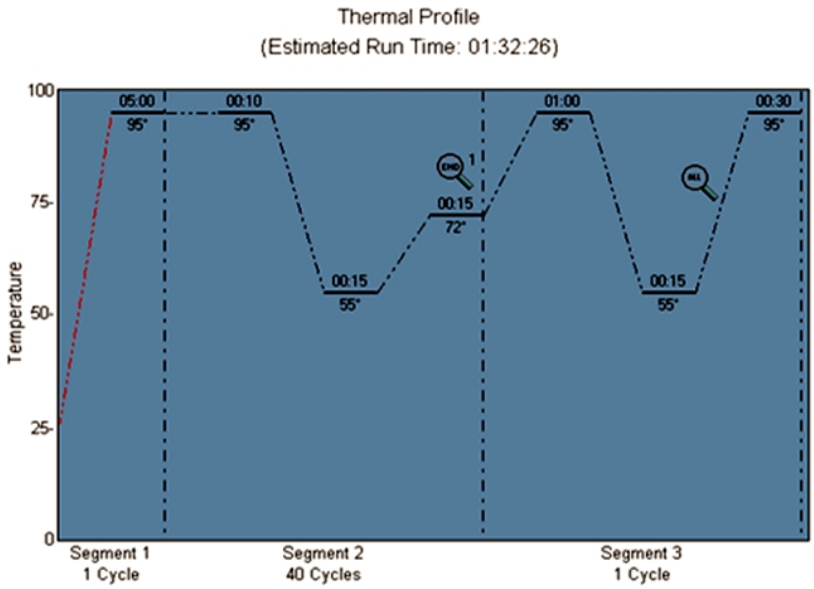


Fig. 4.9 : Thermal profile of qRT-PCR

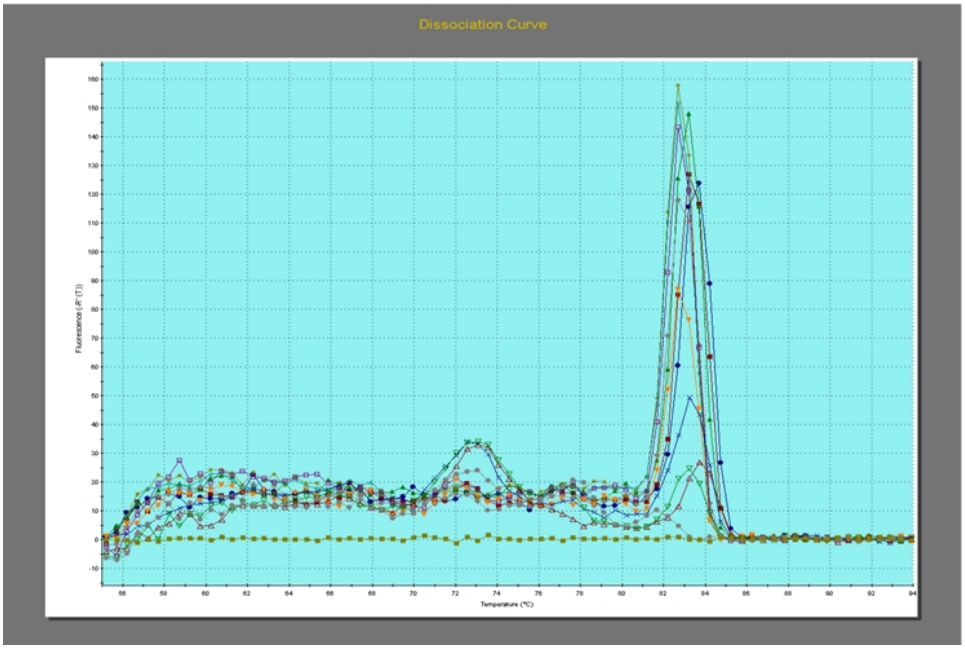


Fig. 4.10: Detection of JE virus in pig blood samples by qRT-PCR based on the dissociation curves plotted with MxProTM QPCR

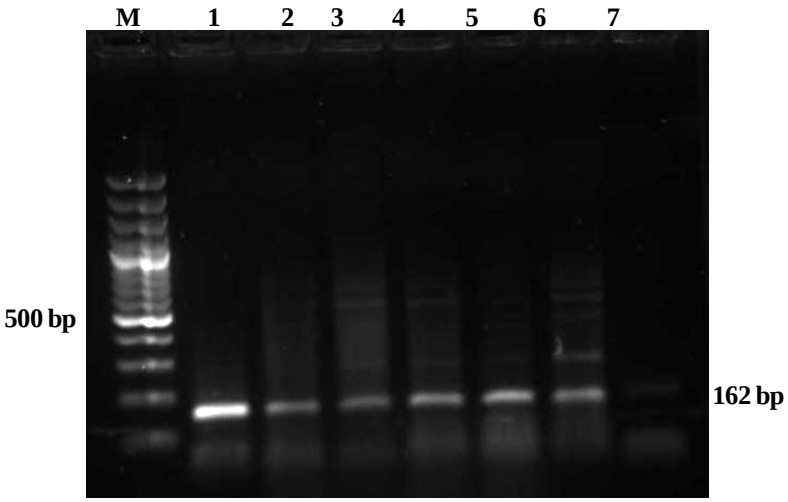


Fig. 4.11: Agarose gel showing the positive qRT-PCR samples

Lane M : 100 bp DNA ladder
Lane 1 : Positive control (162 bp)
Lane 2-6 : positive for JEV

4.2.5 Detection and quantification of JEV in pig blood samples

A total of 426 pig blood samples were screened by qRT-PCR assay. The JE viral load in terms of copy numbers per microlitre of the sample was calculated based on the extrapolation of the 'Ct' (Threshold cycle) values of the samples against the standard curve (Fig. 4.6 & 4.7). JEV was detected and quantified in 18 (4.22%) of pig blood samples (Table 4.1). The viral load was in the range of 4.243×10^9 to 2.53×10^2 copy numbers. The real-time PCR assay could detect the virus in all the 17 blood samples which were earlier detected as positive by RT-PCR. The real-time PCR positive samples were run on agarose gel for confirmation of expected product size of 162 bp (Fig. 4.11).

Table 4.1: Quantification of JE viral load in pig blood samples

Sl. No.	Sample No.	RT-PCR	qRT-PCR	Real-time PCR	
				'Ct' value	Copy number
1	11GS	-	+	29	$2.53x10^2$
2	49	+	+	18	$9.951x10^7$
3	50	+	+	22	$8.51x10^5$
4	M	+	+	13.84	$2.243x10^9$
5	7G	+	+	26	$5.14x10^3$
6	Q	+	+	21	$6.31x10^6$
7	56	+	+	16	$3.74x10^8$
8	3G	+	+	26	$5.43x10^4$
9	55	+	+	28	$5.8x10^2$
10	27	+	+	25	$4.32x10^3$
11	10SG	+	+	13.34	$4.243x10^9$
12	8G	+	+	15	$4.301x10^8$
13	4G	+	+	25	$7.34x10^5$
14	21	+	+	18.48	$9.951x10^7$
15	G	+	+	16	$6.102x10^8$
16	14SG	+	+	24	$4.81x10^4$
17	19SG	+	+	14.71	$1.569x10^9$
18	13S	+	+	17	$9.25x10^8$

4.3 Isolation of Japanese Encephalitis Virus

Two pig blood samples (positive by both RT-PCR and Real-time PCR) were subjected for isolation of JEV using Vero cell line. The JE virus could not be isolated from any of the sample.

4.4 Seroprevalence of JE in pigs by indirect ELISA

The standardized whole JE viral antigen based indirect ELISA was used to study the seroprevalence of Japanese encephalitis in pigs. A total of 426 serum samples that were screened by IgG targeted ELISA, revealed 101 (23.7%) samples as positive for IgG antibodies against JE in pigs (Fig. 4.12 & 4.13). Of the 165 sera samples collected from Piggery farm, IVRI, pork retail markets of Bareilly, Gorakhpur and Balia districts of Uttar Pradesh, 48 (29.09%) samples found positive for JE antibodies. Out of 261 samples collected from Deonar slaughter house, Mumbai, 53 (20.30%) samples found positive for JE (Table 4.2).

Table 4.2: Prevalence of JEV by RT-PCR, Real time PCR and IgG ELISA in pigs

Sl. No.	Sample No.	RT-PCR	Real-time PCR	IgG ELISA
Samples collected from Bareilly, Gorakhpur and Balia districts of Uttar Pradesh				
1	424	-	-	+
2	3	-	-	+
3	7/9 1	-	-	+
4	16/10 3	-	-	+
5	S4	-	-	+
6	10/10 3	-	-	+
7	20/10 2	-	-	+
8	S5	-	-	+
9	25/8 II	-	-	+
10	10/10 2	-	-	+
11	16/10 4	-	-	+
12	29/9 1	-	-	+
13	383	-	-	+
14	23/10 2	-	-	+
15	26/8 1	-	-	+
16	6/9 1	-	-	+

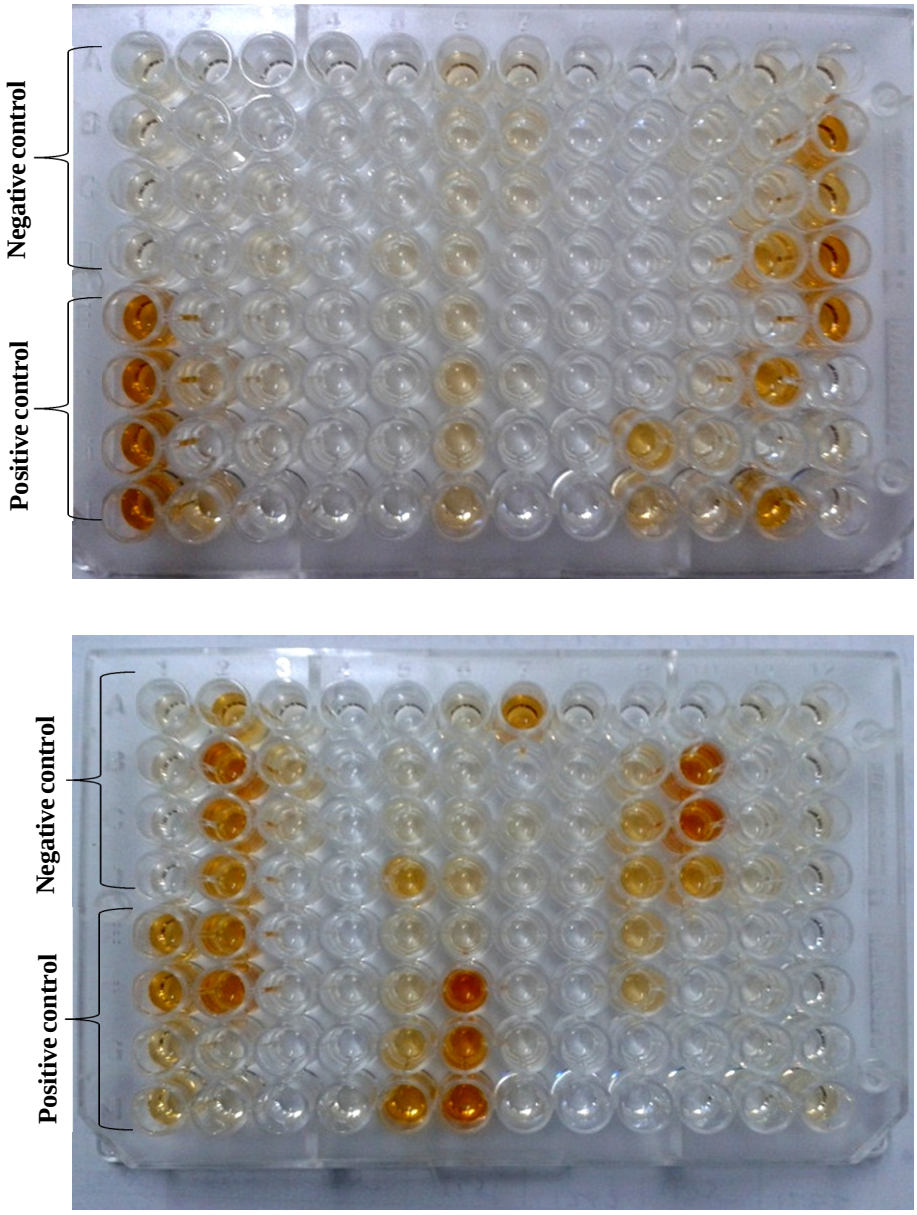


Fig. 4.12 & 4.13:Detection of IgG against JEV in pigs by whole virus antigen based ELISA

Table 4.2: Contd...

Sl. No.	Sample No.	RT-PCR	Real-time PCR	IgG ELISA
17	5/9	-	-	+
18	S2	-	-	+
19	19/10 II	-	-	+
20	20/10 1	-	-	+
21	17/10 1	-	-	+
22	24/10 1	-	-	+
23	23/10 5	-	-	+
24	23/10 1	-	-	+
25	16/10 2	-	-	+
26	18/10	-	-	+
27	23/10 4	-	-	+
28	11/10	-	-	+
29	11GS	-	+	-
30	49	+	+	-
31	20	-	-	+
32	69	-	-	+
33	50	+	+	-
34	F	-	-	+
35	48	-	-	+
36	M	+	+	-
37	53	-	-	+
38	**7G***	**+**	**+**	**+**
39	Q	+	+	-
40	56	+	+	-
41	1G	-	-	+
42	5G	-	-	-
43	47	-	-	+
44	10/10 2	-	-	+
45	**3G***	**+**	**+**	**+**
46	32	-	-	+
47	1	-	-	+
48	59	-	-	+
49	2	-	-	+
50	19/10 1	-	-	+
51	55	+	+	-
52	27	+	+	-

Table 4.2: Contd...

Sl. No.	Sample No.	RT-PCR	Real-time PCR	IgG ELISA
53	19	-	-	+
54	10SG	+	+	-
55	8G	+	+	-
56	16/10 4	-	-	+
57	**4G***	**+**	**+**	**+**
58	21	+	+	-
59	19/10	-	-	+
60	G	+	+	-
61	**14SG***	**+**	**+**	**+**
62	9SG	+	+	-
63	13S	+	+	-
Samples from Deonar slaughter house, Mumbai				
64	M 59	-	-	+
65	M91	-	-	+
66	M40	-	-	+
67	M61	-	-	+
68	M73	-	-	+
69	M57	-	-	+
70	M111	-	-	+
71	M114	-	-	+
72	M70	-	-	+
73	M72	-	-	+
74	M93	-	-	+
75	M14	-	-	+
76	M3	-	-	+
77	M90	-	-	+
78	M45	-	-	+
79	M9	-	-	+
80	M166	-	-	+
81	M221	-	-	+
82	M262	-	-	+
83	M180	-	-	+
84	M254	-	-	+
85	M156	-	-	+
86	M259	-	-	+

Table 4.2: Contd...

Sl. No.	Sample No.	RT-PCR	Real-time PCR	IgG ELISA
87	M236	-	-	+
88	M242	-	-	+
89	M261	-	-	+
90	M154	-	-	+
91	M224	-	-	+
92	M74	-	-	+
93	M232	-	-	+
94	M215	-	-	+
95	M228	-	-	+
96	M219	-	-	+
97	M216	-	-	+
98	M264	-	-	+
99	M159	-	-	+
100	M195	-	-	+
101	M158	-	-	+
102	M151	-	-	+
103	M62	-	-	+
104	M54	-	-	+
105	M89	-	-	+
106	M7	-	-	+
107	M53	-	-	+
108	M8	-	-	+
109	M85	-	-	+
110	M239	-	-	+
111	M247	-	-	+
112	M60	-	-	+
113	M68	-	-	+
114	M170	-	-	+
115	M71	-	-	+
116	M64	-	-	+
Total positive (% prevalence) (n=426)		**17 (3.99%) Blood samples found positive by RT-PCR**	**18 (4.22%) Blood samples found positive by Real time PCR**	**101 (23.7%) of Serum samples found positive by IgG ELISA**

(Sample No. 7G*, 3G*, 4G* and 14SG* from Gorakhpur region tested positive by all the three tests).

4.5 Area wise prevalence of JE by RT-PCR, Real-time PCR and I-ELISA

Out of 30 samples collected and screened from piggery farm, IVRI, Izatnagar revealed seropositivity of 6.66% by I-ELISA, whereas none of the sample was found positive by RT-PCR and Real-time PCR. The prevalence rate was found to be higher in samples of Gorakhpur area i.e., 64.28% (RT-PCR), 71.42% (Real-time PCR) and 35.71% (I-ELISA). In Balia district, the prevalence rate by various tests was 11.11% (RT-PCR), 11.11% (Real-time PCR) and 16.66% (I-ELISA). A seroprevalence of 59.18% (I-ELISA) was observed in samples collected from pig slaughter house, Bareilly. Out of 261 samples collected from Deonar slaughter house, Mumbai, 53 (20.30%) samples found positive for JE by I-ELISA. The area wise prevalence of JE by RT-PCR, Real-time PCR and I-ELISA were depicted in the table 4.3.

Table 4.3: Area wise prevalence of JE by RT-PCR, Real-time PCR and I-ELISA

Area/Farm	Total samples screened	Positive by RT-PCR	Positive by Real-time PCR	Positive by I-ELISA
Piggery farm IVRI, Izatnagar	30	-	-	2 (6.66%)
Gorakhpur area	14	9 (64.28%)	10 (71.42%)	05(35.71%)
Balia district	72	8 (11.11%)	8 (11.11%)	12 (16.66%)
Pig slaughter house, Bareilly	49	-	-	29 (59.18%)
Deonar slaughter house, Mumbai	261	-	-	53 (20.30%)
Prevalence (% positivity) (n=426)	**426**	**17 (3.99%)**	**18 (4.22%)**	**101 (23.7%)**

4.6 Comparison between prevalence of JEV by RT-PCR, Real-time PCR and Indirect ELISA

In the present study, RT-PCR could detect JEV in a total of 17 (3.99%) of the samples. But application of Real-time PCR for screening of pig blood samples revealed 18 (4.22%) samples as positive. With Real-time PCR assay 1 sample found positive which was earlier

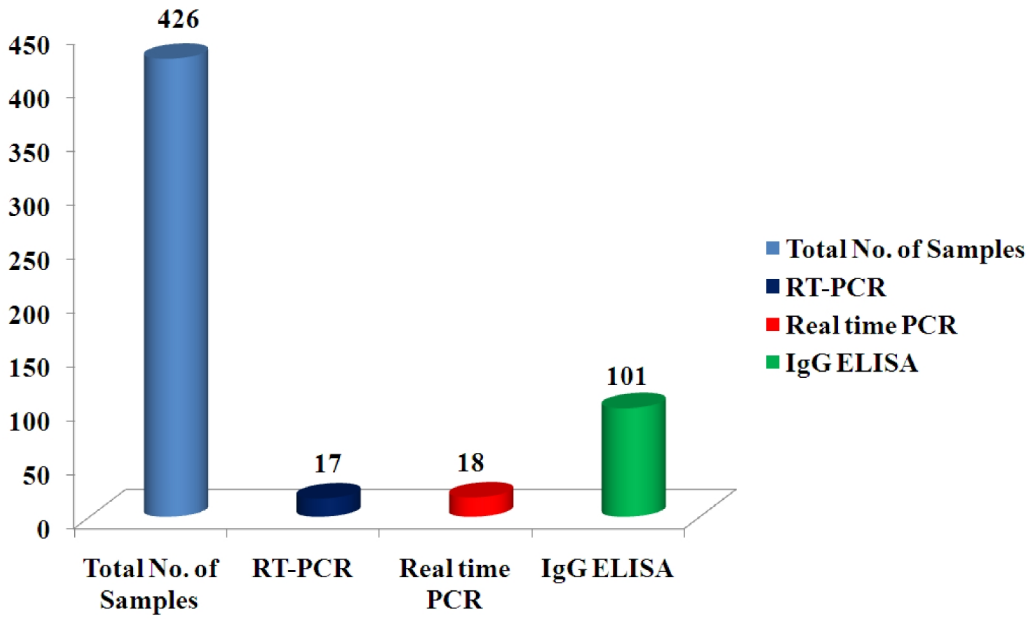


Fig. 4.14 : Prevalence of JE in pig blood and sera samples by different tests

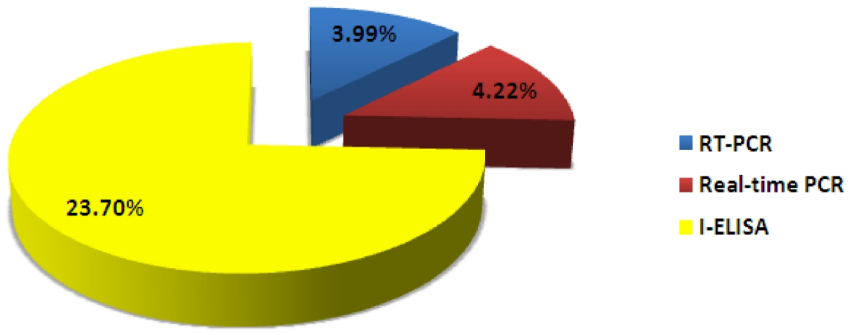


Fig. 4.15: Comparative evaluation of percent prevalence of JEV in pigs by RT-PCR, Real-time PCR and I-ELISA

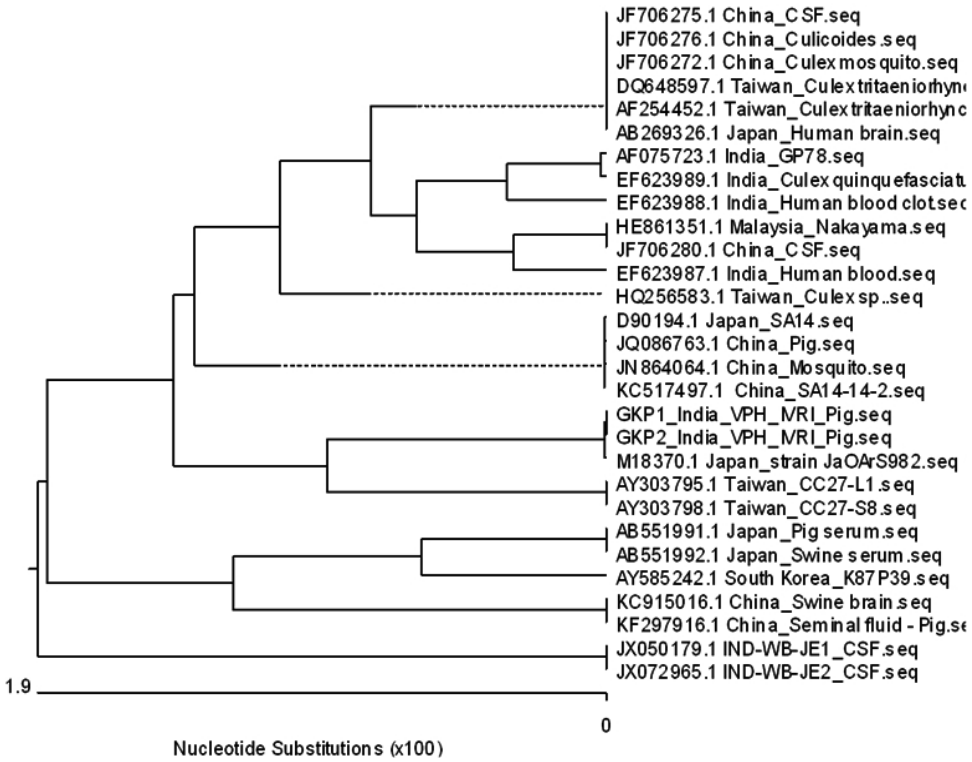


Fig. 4.16: Phylogenetic tree of partial sequences of GKP1_India_IVRI_VPH and GKP2_India_IVRI_VPH (NS3 gene) with reported sequences of JEV

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	
1		97.5	97.5	98.8	100.0	97.5	97.5	97.5	99.4	100.0	98.8	99.4	97.5	98.1	98.1	99.4	99.4	100.0	100.0	100.0	99.4	99.4	99.4	96.9	96.9	99.4	96.3	96.3	98.1	1
2	2.5		100.0	96.3	96.9	96.3	96.3	98.8	98.1	96.9	96.3	96.9	94.4	96.9	96.9	96.3	96.3	96.9	96.9	96.9	96.3	98.1	97.5	96.3	96.3	98.1	97.5	97.5	96.9	2
3	2.5	0.0		96.3	96.9	96.3	96.3	98.8	98.1	96.9	96.3	96.9	94.4	96.9	96.9	96.3	96.3	96.9	96.9	96.9	96.3	98.1	97.5	96.3	96.3	98.1	97.5	97.5	96.9	3
4	1.3	3.8	3.8		98.1	97.5	97.5	98.3	98.1	98.1	98.8	99.4	96.1	96.9	96.9	97.5	97.5	98.1	98.1	98.1	97.5	98.1	97.5	95.1	95.1	98.1	95.1	95.1	96.9	4
5	0.0	2.5	2.5	1.3		97.5	97.5	97.5	99.4	100.0	98.8	99.4	97.5	98.1	98.1	99.4	99.4	100.0	100.0	100.0	99.4	99.4	99.4	96.9	96.9	99.4	96.3	96.3	98.1	5
6	2.5	3.8	3.8	2.5	2.5		100.0	96.3	98.1	96.9	97.5	98.1	95.7	98.1	98.1	96.3	96.3	96.9	96.9	96.9	96.3	98.1	97.5	95.1	95.1	98.1	97.5	97.5	98.1	6
7	2.5	3.8	3.8	2.5	2.5	0.0		96.3	98.1	96.9	97.5	98.1	95.7	98.1	98.1	96.3	96.3	96.9	96.9	96.9	96.3	98.1	97.5	95.1	95.1	98.1	97.5	97.5	98.1	7
8	2.5	1.3	1.3	3.8	2.5	3.8	3.8		98.1	96.9	96.3	96.9	94.4	96.9	96.9	96.3	96.3	96.9	96.9	96.9	96.3	98.1	97.5	96.3	96.3	98.1	97.5	97.5	96.9	8
9	0.6	1.9	1.9	1.9	0.6	1.9	1.9	1.9		98.8	98.1	98.8	96.3	98.8	96.8	98.1	98.1	98.8	98.8	98.8	98.1	100.0	99.4	96.9	96.9	100.0	96.9	96.9	98.8	9
10	0.0	2.5	2.5	1.3	0.0	2.5	2.5	2.5	0.6		98.8	99.4	97.5	98.1	98.1	99.4	99.4	100.0	100.0	100.0	99.4	99.4	99.4	96.9	96.9	99.4	96.3	96.3	98.1	10
11	1.3	3.8	3.8	1.3	1.3	2.5	2.5	3.8	1.9	1.3		99.4	96.9	96.9	96.9	98.8	97.5	98.1	98.1	98.1	98.8	98.1	97.5	95.1	95.1	98.1	95.1	95.1	96.9	11
12	0.6	3.2	3.2	0.6	0.6	1.9	1.9	3.2	1.3	0.6	0.6		97.5	97.5	97.5	98.1	96.1	98.8	98.8	98.8	98.1	98.8	98.1	95.7	95.7	98.8	95.7	95.7	97.5	12
13	1.3	3.9	3.9	0.0	1.3	2.6	2.6	3.9	1.9	1.3	1.3	0.6		96.9	96.9	98.1	98.1	98.7	98.7	98.7	98.1	98.1	98.1	95.6	95.6	98.1	95.0	95.0	96.9	13
14	1.9	3.2	3.2	3.2	1.9	1.9	1.9	3.2	1.3	1.9	3.2	2.5	3.2		100.0	96.9	96.9	97.5	97.5	97.5	96.9	98.8	98.1	95.7	95.7	98.8	96.9	96.9	100.0	14
15	1.9	3.2	3.2	3.2	1.9	1.9	1.9	3.2	1.3	1.9	3.2	2.5	3.2	0.0		96.9	96.9	97.5	97.5	97.5	96.9	98.8	98.1	95.7	95.7	98.8	96.9	96.9	100.0	15
16	0.6	3.2	3.2	1.9	0.6	3.2	3.2	3.2	1.3	0.6	0.6	1.3	1.9	2.5	2.5		96.8	99.4	99.4	99.4	100.0	98.8	98.8	96.3	96.3	98.8	95.7	95.7	97.5	16
17	0.6	3.2	3.2	1.9	0.6	3.2	3.2	3.2	1.3	0.6	1.9	1.3	1.9	2.5	2.5	1.3		99.4	99.4	99.4	98.8	98.8	98.8	97.5	97.5	98.8	95.7	95.7	97.5	17
18	0.0	2.5	2.5	1.3	0.0	2.5	2.5	2.5	0.6	0.0	1.3	0.6	1.3	1.9	1.9	0.6	0.6		100.0	100.0	99.4	99.4	99.4	96.9	96.9	99.4	96.3	96.3	98.1	18
19	0.0	2.5	2.5	1.3	0.0	2.5	2.5	2.5	0.6	0.0	1.3	0.6	1.3	1.9	1.9	0.6	0.6	0.0		100.0	99.4	99.4	99.4	96.9	96.9	99.4	96.3	96.3	98.1	19
20	0.0	2.5	2.5	1.3	0.0	2.5	2.5	2.5	0.6	0.0	1.3	0.6	1.3	1.9	1.9	0.6	0.6	0.0	0.0		99.4	99.4	99.4	96.9	96.9	99.4	96.3	96.3	98.1	20
21	0.6	3.2	3.2	1.9	0.6	3.2	3.2	3.2	1.3	0.6	0.6	1.3	1.9	2.5	2.5	0.0	1.3	0.6	0.6	0.6		98.8	98.8	96.3	96.3	98.8	95.7	95.7	97.5	21
22	0.6	1.9	1.9	1.9	0.6	1.9	1.9	1.9	0.0	0.6	1.9	1.3	1.9	1.3	1.3	1.3	1.3	0.6	0.6	0.6	1.3		99.4	96.9	96.9	100.0	96.9	96.9	98.8	22
23	0.6	1.9	1.9	1.9	0.6	1.9	1.9	1.9	0.0	0.6	1.9	1.3	1.9	1.3	1.3	1.3	1.3	0.6	0.6	0.6	1.3	0.0		97.5	97.5	100.0	96.9	96.9	98.8	23
24	3.2	3.2	3.2	4.5	3.2	4.5	4.5	3.2	2.5	3.2	4.5	3.9	4.6	3.9	3.9	3.9	2.5	3.2	3.2	3.2	3.9	2.5	2.5		100.0	97.5	95.7	95.7	96.3	24
25	3.2	3.2	3.2	4.5	3.2	4.5	4.5	3.2	2.5	3.2	4.5	3.9	4.6	3.9	3.9	3.9	2.5	3.2	3.2	3.2	3.9	2.5	2.5	0.0		97.5	95.7	95.7	96.3	25
26	0.6	1.9	1.9	1.9	0.6	1.9	1.9	1.9	0.0	0.6	1.9	1.3	1.9	1.3	1.3	1.3	1.3	0.6	0.6	0.6	1.3	0.0	0.0	2.5	2.5		96.9	96.9	98.8	26
27	3.9	2.5	2.5	5.2	3.9	2.5	2.5	2.5	3.2	3.9	5.2	4.5	5.3	3.2	3.2	4.5	4.5	3.9	3.9	3.9	4.5	3.2	3.2	4.5	4.5	3.2		100.0	96.9	27
28	3.9	2.5	2.5	5.2	3.9	2.5	2.5	2.5	3.2	3.9	5.2	4.5	5.3	3.2	3.2	4.5	4.5	3.9	3.9	3.9	4.5	3.2	3.2	4.5	4.5	3.2	0.0		96.9	28
29	1.9	3.2	3.2	3.2	1.9	1.9	1.9	3.2	1.3	1.9	3.2	2.5	3.2	0.0	0.0	2.5	2.5	1.9	1.9	1.9	2.5	1.3	1.3	3.9	3.9	1.3	3.2	3.2		29
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	

1 AB269326.1 Japan_Human brain.seq
2 AB551991.1 Japan_Pig serum.seq
3 AB551992.1 Japan_Swine serum.seq
4 AF075723.1 India_GP78.seq
5 AF254452.1 Taiwan_Culex tritaeniorhynch
6 AY303795.1 Taiwan_CC27-L1.seq
7 AY303798.1 Taiwan_CC27-S8.seq
8 AY585242.1 South Korea_K87P39.seq
9 D90194.1 Japan_SA14.seq
10 DQ648597.1 Taiwan_Culex tritaeniorhynch
11 EF623987.1 India_Human blood.seq
12 EF623988.1 India_Human blood clot.seq
13 EF623989.1 India_Culex quinquefasciatus
14 GKP1_India_VPH_IVRI_Pig.seq
15 GKP2_India_VPH_IVRI_Pig.seq
16 HE861351.1 Malaysia_Nakayama.seq
17 HQ256583.1 Taiwan_Culex sp..seq
18 JF706272.1 China_Culex mosquito.seq
19 JF706275.1 China_CSF.seq
20 JF706276.1 China_Culicoides.seq
21 JF706280.1 China_CSF.seq
22 JN864064.1 China_Mosquito.seq
23 JQ086763.1 China_Pig.seq
24 JX050179.1 IND-WB-JE1_CSF.seq
25 JX072965.1 IND-WB-JE2_CSF.seq
26 KC517497.1 China_SA14-14-2.seq
27 KC915016.1 China_Swine brain.seq
28 KF297916.1 China_Seminal fluid - Pig.se
29 M18370.1 Japan_strain JaOArS982.seq

Fig. 4.17: Nucleotide percent identities of NS3 gene sequence of GKP1_India_IVRI_VPH and GKP2_India_IVRI_VPH with reported sequences of JEV

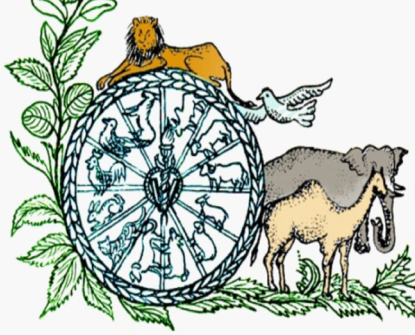
found negative by RT-PCR and this is the first report of application of Real-time PCR for screening of pig blood samples for JEV. While application of I-ELISA for screening of pig sera samples revealed 23.7% seropositivity (Fig. 4.14 & 4.15). Only 4 (Sample No. 7G*, 3G*, 4G* and 14SG*) samples which were collected from Gorakhpur district were found positive by all the three tests *Viz.*, RT-PCR, Real-time PCR and I-ELISA (Table 4.2).

4.7 Phylogenetic analysis of NS3 gene sequences

The NS3 gene sequences of the selected JEV positive clones were sequenced by out sourcing to Chromous Biotech Ltd, Bangalore. The NS3 gene nucleotide sequence homology was determined with previously reported sequences and per cent identity matrix was determined by CLUSTAL W of MegAlign (Lasergene DNA Star) software. Based on the deduced nucleotide sequences, phylogenetic dendrograms and nucleotide percent identity (Fig. 4.17) for GKP1_India_IVRI_VPH and GKP2_India_IVRI_VPH were analyzed with other previously reported sequences of different countries (Fig. 4.16).

The GKP1_India_IVRI_VPH and GKP2_India_IVRI_VPH exhibited maximum nucleotide sequence identity of 100% with Japan JEV strain JaOArs982 (M18370), 98.8% similarity with mosquito isolate of JEV from China (JN 864064) and SA 14-14-2 strain of China (KC517497). It exhibited 98.1% similarity with pig isolate of China (JQ086763), 97.5% with Culex mosquito isolate from China (JF706272) and human isolate from China (JF706275). A 96.9% identity with JEV isolate of *Culex* mosquito from Taiwan (HQ25658) and Swine brain isolate (KC915016). Similarly, 98.1% identity with human isolate from Japan (AB269326) and 96.6% with South Korean strain K87P39 (AY585242). It shared about 95.7% identity with human isolate of JEV from West Bengal, India (JX050179 and JX072965).

Discussion



5 DISCUSSION

Japanese encephalitis (JE) is a vector-borne viral disease that occurs in South Asia, Southeast Asia, East Asia, and the Pacific (Solomon, 2006). Japanese encephalitis virus (JEV) is a major cause of viral encephalitis in Southeast Asia; >50,000 cases are reported annually (Mackenzie *et al*., 2007). The virus exists in a zoonotic transmission cycle among mosquitoes, pigs, bats, and water birds belonging to the family Ardeidae (cattle egrets and pond herons). Humans become infected when bitten by an infected mosquito and are a dead-end host because of low viremia, preventing the virus from being transmitted further (Solomon, 2004). Rapid globalization, population explosion, changes in global climatic condition, industrialization and deforestation, all seem to correlate with the spread of the virus into newer territories. Today, with approximately three billion people living in the JE endemic region, there are estimated 35,000–50,000 cases and 10,000–15,000 deaths annually, thereby making JE one of the most dreaded vector-borne viral encephalitis in the world (Tsai, 2000). It is primarily a disease of children (5-15 years) with neurological sequele and death. It causes acute encephalitis with fatality rates ranging from 20% to as high as 50% (Burke and Leake, 1988; Vaughn and Hoke, 1992). In 1978, cases were reported from 21 states and union territories, and from then onwards till 2007 there have been 103,389 reported cases of JE in India that has led to 33,729 deaths (Dhillon and Raina, 2008). Approximately 597,542,000 people in India live under the JE-endemic regions and there are 1500–4000 reported cases every year (Kabilan et al., 2004a). These figures are based on total reported cases and it is quite possible that several cases may go unreported and hence the actual magnitude of the threat of JE may be considerably higher both in the India as well as the global context.

The laboratory diagnosis routinely used for JEV infection is based on four basic types of assays; serology, virus isolation, immunocytochemistry, and molecular techniques. Serologically, JEV infection can be detected by immunoglobulin M (IgM) and IgG capture enzyme-linked immunosorbent assay (ELISA) (Burke *et al.*, 1982; Endy and Nisalak, 2002; Solomon *et al.*, 1998). Virus isolation from clinical and surveillance samples has generally been unsuccessful, owing to the low level of transient viremia associated with the disease process, and also requires viable virus in samples. One of the recent advances in the diagnostics is the use of molecular techniques such as polymerase chain reaction (PCR), Real-time PCR, reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) etc. The reverse transcription polymerase chain reaction (RT-PCR) and Real-time RT-PCR has been reported by several workers for quick and sensitive detection of JEV RNA from clinical samples of human and animals (Shirato *et al.*, 2003; Yang *et al.*, 2005; Parida *et al.*, 2006a; Sapkal *et al.*, 2007; Santhosh *et al.*, 2007; Saxena *et al.*, 2009).

Japanese encephalitis (JE) is caused by the Japanese encephalitis virus (JEV). It is a major public health problem in Asia. Swine is a viral amplifier, and thus plays a critical role in JEV transmission to human beings. Thus, development of a rapid method for early JEV detection in swine is required, as a step to control viral spread. In India, limited work has been carried out on prevalence of JE in pigs, especially application of molecular techniques *viz.*, RT-PCR and Real-time PCR in determining the prevalence of JEV in pigs. By keeping the above facts in view, there is an urgent need to know the prevalence of Japanese encephalitis in case of pigs in endemic regions of India. Therefore, the present study was undertaken to standardize reverse transcriptase-PCR (RT-PCR) and Real-time RT-PCR assay for the rapid detection of Japanese encephalitis in pigs; to study the prevalence of JEV in pigs by Indirect enzyme linked immunosorbent assay, RT-PCR and Real-time PCR assays.

5.1 Standardization of RT-PCR

The RT-PCR standardized for detection of JEV using standard JEV RNA extracted from GP78 infected Vero cell line. The RT-PCR was standardized for envelope 'E' gene using self designed primers and this gene segment yielded expected product size of 474 bp. Out of 426 pig blood samples, 17 (3.99%) were positive for JEV RNA, as the cDNA from

these samples when amplified by PCR revealed the expected product of 474 bp. With the available data when the results were analyzed further revealed that, only the samples from Uttarpradesh tested positive by RT-PCR i.e., out of 165 pig blood samples 17 (10.30%) for JEV RNA whereas samples collected from Deonar Slaughte House, Mumbai tested negative for JEV RNA by RT-PCR assay.

Saxena *et al*. (2009) had suggested that the use of RT-PCR in serum samples during the early days of JEV infection in humans may be helpful in confirming diagnosis in those cases which were negative for JEV-specific IgM antibodies in both serum and CSF samples.

Swami *et al*. (2008) felt, the need for carrying out RT-PCR in CSF samples, instead of IgM antibody detection, for the early detection of JEV.

Sapkal *et al*. (2007) reported detection by RT-PCR and isolation of JEV from blood clots collected during acute phase of infection in human beings. JEV isolation was attempted from white blood cells (WBCs) separated from blood clots of 12 human patients (9 IgM positive and 3 negative) by serial co-culturing with phytohemagglutinin P-stimulated peripheral blood mononuclear leukocytes (PBMCs) obtained from pre-screened JEV sero-negative healthy individuals. JEV was isolated from two IgM-positive blood clots. Isolate 014178 was detected in WBCs and in the first passage of PBMCs by ELISA and reverse transcriptase-polymerase chain reaction. Isolate 014173 was detectable only after a second passage in PBMC co-culture. This was the first report on isolation of JEV from patient blood clots.

Whereas in the present study, PBMCs could not be isolated from pig blood samples due to the lack of logistics for isolation of PBMCs at the site of sample collection. This might be a reason for low prevalence of JEV by RT-PCR in pig blood samples.

The field of JEV diagnosis has improved remarkably with the application of molecular diagnostic systems, such as reverse transcriptase–polymerase chain reaction (RT-PCR), for early detection and identification of JEV in clinical samples. Several RT-PCR systems have been reported for detection of JEV envelope gene in various biological samples such as infected cell cultures, Aedes larvae, mosquitoes, pigs and mouse blood (Igarashi *et al*., 1994; Paranjpe and Banerjee, 1998; Parida *et al*., 2006a).

Jeonga *et al.* (2011) reported a novel RT-PCR kit with an internal positive control with automated RNA extraction system for screening of JEV in field-collected mosquitoes. This may offer an alternative approach for JEV surveillance and laboratory diagnosis in the circumstances where real-time RT-PCR system is not available.

Diagnosis of JEV infection is mainly based on serological, viral culture and molecular approaches. Serological methods are simple and feasible. Virus isolation is a definitive diagnosis for JEV infection, however, this assay is usually unavailable owing to less sensitive and more time consuming. RT-PCR assays for detection of specific genomic sequence of JEV have been showed high sensitivity and specificity (Swami *et al.*, 2008). However, these assays compared with serologic tests are more time-consuming and require expensive and sophisticated equipments. Most of cases of JE occur in rural areas, where routine RT-PCR diagnostic facilities are limited.

5.2 Detection and quantification of JEV by Real-time PCR assay

The detection as well as quantification of Japanese encephalitis viral load in pig blood samples was carried out by quantitative RT-PCR assay using QuantiFast Syber Green PCR kit (Quiagen) by following the protocol of Santhosh *et al.* (2007) and targeting the NS3 gene with necessary modifications. The quantitative RT-PCR assays are slowly replacing conventional RT-PCR assays for simultaneous detection and quantification of virus and viral load respectively. In the present study, JEV was detected and quantified in 18 (4.22%) of pig blood samples. The viral load was in the range of $4.243x10^9$ to 2.53×10^2 copy numbers per reaction. The Real-time PCR assay could detect the virus in 18 blood samples in which 17 were earlier detected as positive by RT-PCR. Thus, real-time PCR was found more sensitive than the conventional RT-PCR. This seems to be the first report on the application of Real-time PCR for detection as well as quantification of JE viral load in pig blood samples.

Sapkal *et al.* (2007) reported one-step SYBR Green I-based real-time RT-PCR assay for rapid detection as well as quantitation of Japanese encephalitis virus (JEV) in acute-phase patient CSF samples by targeting the NS3 gene. The applicability of Real-time PCR assay for clinical diagnosis was validated with 32 suspected acute-phase CSF samples of Gorakhpur epidemic, India, 2005.

Chen *et al.* (2011) developed and evaluated a reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for detecting Japanese encephalitis virus (JEV). The sensitivity of the JEV RT-LAMP assay was in concordance with that of real-time RT-PCR and 10-fold more sensitive than that of conventional RT-PCR, with the detection limit of 24 copies/µl.

In the past decades, several nucleic acid-based amplification methods for detection of JEV infection have been established, which including conventional RT-PCR, nested RT-PCR, probe real-time RT-PCR, dye real-time RT-PCR and LAMP RT-PCR (Huang *et al.*, 2004; Yang *et al.*, 2004; Saxena *et al.*, 2009). Compared to conventional RT-PCR and nested RT-PCR, real-time RT-PCR has engendered wider acceptance due to its improved rapidity, sensitivity and the reduced risk of laboratory contamination. Real-time RT-PCR method has several advantages over conventional PCR. Firstly, real-time RT-PCR is a more rapid and sensitive test as compared to conventional RT-PCR. The second advantage of the closed one-tube real-time RT-PCR is that it is less likely to produce false positive by contamination during sample preparation. There are currently five main chemistries for the detection of PCR product during real-time PCR (Mackay *et al*., 2002).

5.3 Isolation of Japanese Encephalitis Virus

Attempts were made for isolation of JE virus from two pig blood samples (positive by both RT-PCR and Real-time PCR) were subjected for isolation of JEV using Vero cell line. But we could not isolate the virus from any of the sample. The virus isolation is difficult in case of JEV, even in the best laboratory facility, probably because of low level of viremia and the rapid development of neutralizing antibodies (Solomon *et al*., 2004 and Parida *et al*., 2006b). The chances of virus isoaltion are more if the clinical samples are colleced in very acute period of infection (with in 2 days of infection) as neutralizing antibodies to JEV are developed very rapidly (Perida *et al.*, 2006b). The isolation results of present study are in agreement with the JEV isolation results of Kolhe (2008).

However, isolation of virus from clinical specimens is generally considered a rare occurrence (Shope and Sather, 1979) probably because of low viral titers, rapid production of neutralizing antibodies, and the logistic difficulty in transportation of specimens in developing

countries and frequent freezing and thawing of clinical material (Mohanrao *et al.,* 1983; Leake *et al.,* 1986).

5.4 Seroprevalence of JE in pig by indirect ELISA

The whole viral antigen based standardized indirect ELISA was used to study the seroprevalence of Japanese encephalitis in pigs. A total of 426 serum samples were screened by IgG targeted ELISA, revealed 101 (23.76%) samples found positive for IgG antibodies against JE in pigs. Of the 165 sera samples collected from Piggery farm, IVRI, pork retail markets of Bareilly, Gorakhpur and Balia districts of Uttarpradesh, 48 (29.09%) samples found positive for JE antibodies. Out of 261 samples collected from Deonar slaughter house, Mumbai, 53 (20.30%) samples found positive for JE. The whole viral antigen based ELISA had a sensitivity, specificity, efficacy, positive predictive value and negative predictive value of 86.6%, 100%, 91.66%, 100% and 81.81% respectively (Rawat, 2013).

The results are comparable with a study by Yang *et al*. (2006), who have reported 21.7% (156/720) of JEV seroprevalence by whole viral antigen based I-ELISA in pigs. But, the seroprevalence was relatively higher in Gyeongnam (45%, 81/180) than the other regions (12.2% (11/90) in Jeju, 12.6% (34/270) in Gyeonggi, 16.7% (30/180) in Jeonbuk, respectively) in Korea.

Higher sero-prevalence of JE in swine population was observed from all the area of collection and such prevalence rate is obvious as swine plays important role in JEV amplification and harboring infection for longer periods of time (Geevergheese *et al.,* 1991 and Solomon *et al.,* 2003). Johanna *et al*. (2013) reported 43 female pigs from the urban households and 30 out of the 31 from rural households within Can Tho City, Vietnam were positive by IgG ELISA giving an overall seroprevalence of 99%.

The prevalence rate of the present study is in similar with several reports from various parts of India have reported varying degrees of prevalence of JE in swine *viz.*, Karnataka-47.22% (Geevergheese *et al.,* 1987a), Bareilly and adjoining districts-40% (Mall *et al.,* 1995), Haryana-18% (Nagaleelavathi *et al.,* 2008), Tamil Nadu-26.4% (Kumanan *et al.,* 2002) and Chandigarh-30.03% (Ratho *et al.,* 1999). The overall prevalence of present study is in agreement with the findings of Kolhe (2008), who recorded a prevalence of 28.89% by

indirect IgG ELISA using JEV whole viral antigen. Rawat (2013) reported 33% seropositivity in pigs.

5.5 Area wise prevalence of JE by RT-PCR, Real-time PCR and I-ELISA

Out of 30 samples collected and screened from piggery farm, IVRI, Izatnagar revealed seropositivity of 6.66% by I-ELISA, whereas none of the sample found positive by RT-PCR and Real-time PCR. The prevalence rate was found to be higher in samples of Gorakhpur area i.e., 64.28% and 71.42% by RT-PCR and real-time PCR respectively and seropositivity of 35.71%. In Balia district, the prevalence rate was 11.11% by RT-PCR and real-time PCR and 16.66% seropositivity by I-ELISA. A seroprevalence of 59.18% was found in samples collected from pig slaughter house, Bareilly. Out of 261 samples collected from Deonar slaughter house, Mumbai, 53 (20.30%) samples found positive for JE by I-ELISA.

5.6 Comparison between prevalence of JEV by RT-PCR, Real-time PCR and Indirect ELISA

In the present study, RT-PCR could detect JEV in a total of 17 (3.99%) of the samples. But application of Real-time PCR for screening of pig blood samples revealed 18 (4.22%) samples found positive. With Real-time PCR assay 1 sample found positive which was earlier found negative by RT-PCR and this is the first report of application of Real-time PCR for screening of pig blood samples for JEV. While application of I-ELISA for screening of pig sera samples revealed 23.7% seropositivity. Only 4 samples which were collected from Gorakhpur district found positive by all the three tests *viz.*, RT-PCR, Real-time PCR and I-ELISA. This may be due to increase in mosquito population, viral activity and increase in production of antibodies against the JE infection in pigs and these samples were collected and screened during the period December month. The results are in agreement with published reports confirming Real-time PCR to be more sensitive than RT-PCR (Santhosh *et al*., 2007; Parida *et al*., 2006a; Chen *et al*., 2011).

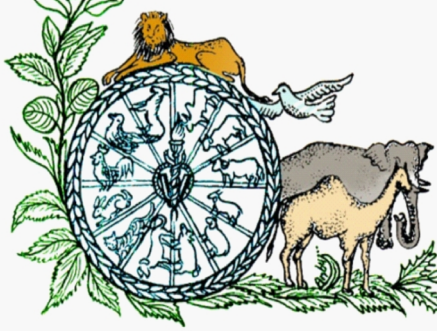
5.7 Phylogenetic analysis of NS3 gene sequences

In the present study, GKP1_India_IVRI_VPH and GKP2_India_IVRI_VPH exhibited maximum nucleotide sequence identity of 100% with Japan JEV strain JaOArs982

(M18370), 98.8% similarity with mosquito isolate of JEV from China (JN 864064) and SA-14-14-2 strain of China (KC517497). It exhibited 98.1% similarity with pig isolate of China (JQ086763), 97.5% with Culex mosquito isolate from China (JF706272) and human isolate from China (JF706275). A 96.9% identity with JEV isolate of *Culex* mosquito from Taiwan (HQ25658) and Swine brain isolate (KC915016). Similarly, 98.1% identity with human isolate from Japan (AB269326) and 96.6% with South Korean strain K87P39 (AY585242). It shared about 95.7% identity with two human isolates of JEV from West Bengal, India (JX050179 and JX072965).

The present study revealed that the reported NS3 gene sequences of GKP1_India_IVRI_VPH and GKP2_India_IVRI_VPH were seems to be originated from China/Japan/Taiwan/Korea.

Summary and Conclusions



6 SUMMARY AND CONCLUSIONS

The Japanese encephalitis virus is the single largest cause of viral encephalitis in the world with an estimated 50,000 cases and 10,000 deaths annually. JEV is a member of the genus *Flavivirus* (family *Flaviviridae*) that is transmitted between birds, pigs, and some other domestic animals by *Culex* mosquitoes. JE is a mosquito-borne arboviral infection caused by a flavivirus transmitted by anthropophilic rice-field mosquitoes of the *Culex* species (primarily the *Culex tritaeniorhynchus* and *Culex vishnui* group). Amplifying hosts, chiefly domestic pigs, act as intermediaries in transmitting the virus. The disease is maintained in nature by complex cycle that involves pig as amplifying host, ardeid birds as reservoirs and mosquito as vector. There are two transmission cycles of JEV in nature viz., pig-mosquioto-pig and bird-mosquito-bird. Pigs are the most important reservoirs. Though they do not manifest the disease, they develop very high titers of virus in circulating blood and infect mosquitoes. Thus pigs are the amplifying hosts. Presence of swine population in the peridomestic environment of JE enzootic areas greatly increases the risk of human infections.

Rapid globalization, population explosion, changes in global climatic condition, industrialization and deforestation, all seem to correlate with the spread of the virus into newer territories. JE is likely to remain as an important public health problem in the 21st century. The post-monsoon outbreak of JE is a common feature and outbreaks have been regularly reported from most parts of India since the mid-1950s. The geographical expansion of JEV ranges from the East (Calcutta, West Bengal), North (Uttar Pradesh) to South (Vellore, Tamil Nadu) of the Indian subcontinent. The virus has already established endemicity in different pockets of

the country. The present study was therefore designed with the objective to standardize reverse transcriptase-PCR (RT-PCR) and real-time RT-PCR assay for the rapid detection of Japanese encephalitis in pigs; to study the prevalence of JEV in pigs by Indirect enzyme linked immunosorbent assay, RT-PCR and Real-time PCR assays.

In the present study, a total 852 samples (comprising 426 blood and 426 serum from 426 pigs) were collected from domestic pigs from various regions of Uttar Pradesh (Bareilly, Gorakhpur, Ballia) and Deonar Slaughter House, Mumbai, Maharashtra.

RT-PCR was standardized for detection of JEV using standard JEV RNA (GP78) extracted from infected Vero cell line. The RT-PCR was standardized for envelope 'E' gene using self designed primers and this gene segment yielded expected product size of 474 bp. Out of 426 pig blood samples, 17 (3.99%) were positive for JEV RNA, as the cDNA from these samples when amplified by PCR revealed the expected product of 474 bp. All the positive samples were collected from Uttar Pradesh and none of the samples collected from Deonar, Slaughter House, Mumbai were positive for JEV by this assay. With the available data when analyzed further indicated that, samples from Uttar Pradesh only shown positive by RT-PCR i.e., out of 165 pig blood samples 17 (10.30%) were positive for JEV RNA.

The detection as well as quantification of Japanese encephalitis viral load in the pig blood samples was carried out by Quantitative Real-time PCR assay by using the primers against the NS3 gene of JEV, with amplification product of 162 bp in length. A total of 426 pig blood samples were screened by qRT-PCR assay. The JE viral load in terms of copy numbers per microlitre of the sample was calculated based on the extrapolation of the 'Ct' values of the samples against the standard curve. JEV was detected and quantified in 18 (4.22%) of pig blood samples. The viral load was in the range of 4.243×10^9 to 2.53×10^2 copy numbers per reaction. The Real-time PCR assay could detect the virus in 17 blood samples which were earlier detected as positive by RT-PCR. Thus, it was found to be more sensitive than conventional RT-PCR.

Attempts were made for isolation of JEV from two positive blood samples (positive by both RT-PCR and Real-time PCR) were subjected for isolation of JEV using Vero cell

line. The JE virus could not be isolated from any of the sample. It may be probably because of low level of viremia and the rapid development of neutralizing antibodies.

The whole viral antigen based indirect ELISA was used to study the seroprevalence of Japanese encephalitis in pigs. A total of 426 serum samples were screened by IgG targeted ELISA, revealed 101 (23.7%) samples found positive for IgG antibodies against JE in pigs. Of the 165 sera samples collected from Piggery farm, IVRI, pork retail markets of Bareilly, Gorakhpur and Balia districts of Uttar Pradesh, 48 (29.09%) samples found positive for JE antibodies. Out of 261 samples collected from Deonar slaughter house, Mumbai, 53 (20.30%) samples found positive for JE.

Out of 30 samples collected and screened from piggery farm, IVRI, Izatnagar revealed seropositivity of 6.66% by I-ELISA. The prevalence rate was found to be higher in samples of Gorakhpur area i.e., 64.28%, 71.42% by RT-PCR and Real-time PCR respectively and 35.71% by I-ELISA. In Balia district, the prevalence rate was 11.11% by both the tests (RT-PCR and Real-time RT-PCR) and 16.66% by whole viral antigen based I-ELISA. A seroprevalence of 59.18% and 20.30% was found in samples collected from pig slaughter house, Bareilly and samples from Deonar, slaughter house, Mumbai respectively.

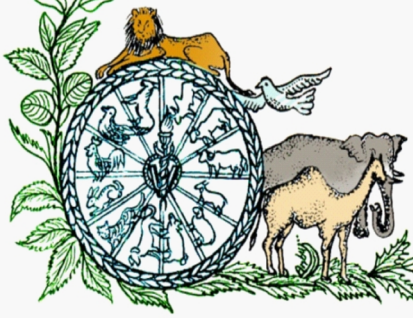
In the present study, the phylogenetic analysis of NS3 gene sequences of GKP1_India_IVRI_VPH and GKP2_India_IVRI_VPH revealed maximum nucleotide sequence identity of 100% with Japan JEV strain JaOArs982 (M18370), 98.8% similarity with mosquito isolate of JEV from China (JN 864064) and SA 14-14-2 strain of China (KC517497). It shared about 95.7% identity with two human isolates of JEV from West Bengal, India (JX050179 and JX072965).

Thus, the present investigation clearly reveals the presence of JEV in pigs through application of RT-PCR, Real-time RT-PCR and indirect ELISA. The JEV specific NS3 gene targeted real-time RT-PCR assay in this study also enable determination of the viral load in pig blood samples. The SYBR Green dye based real-time RT-PCR assay described here for detection and quantitation of JE virus has been shown to be a simple, sensitive and rapid approach for surveillance and epidemiological studies. These features make it an excellent tool

for laboratory detection of JEV in pig blood samples. The indirect ELISA, which was developed in Division of Veterinary Public Health was found to be useful for serodiagnosis of JE in pigs.

Systematic approach is the need of hour, with the joint efforts of public health veterinarians, molecular biologists, public health specialists, drug developers, policy makers and local population to combat against the virus. However, there is indeed a role for integration between veterinary and public health authorities in determining the epidemiology of infection and prospective surveillance for JEV which in turn should help in designing control measures.

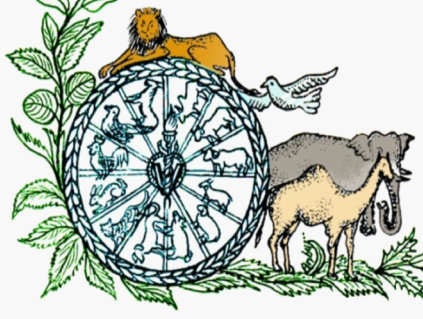
Mini Abstract



7 MINI ABSTRACT

The present investigation was designed with the aim to standardize reverse transcriptase-PCR (RT-PCR) and real-time RT-PCR assay for the rapid detection of Japanese encephalitis virus in pigs and to study the prevalence of JEV in pigs by Indirect enzyme linked immunosorbent assay, RT-PCR and Real-time PCR assays. RT-PCR was standardized for detection of JEV in pig blood samples by targeting envelope 'E' gene. The detection as well as quantification of Japanese encephalitis viral load in the pig blood samples was carried out by quantitative real-time PCR assay by using the primers targeting the NS3 gene of JEV, with amplification product of 162 bp. Out of 426 pig blood samples, 17 (3.99%) were positive for JEV RNA, as the cDNA from these samples when amplified by PCR revealed the expected product of 474 bp. All the RT-PCR positive samples were collected from Uttar Pradesh and none of the samples collected from Deonar, Slaughter House, Mumbai were positive for JEV by RT-PCR assay. A total of 426 pig blood samples were screened by qRT-PCR assay. JEV was detected and quantified in 18 (4.22%) of pig blood samples. The viral load was in the range of 4.243×10^9 to 2.53×10^2 copy numbers per reaction. The Real-time PCR assay could detect the virus in 17 blood samples which were earlier detected as positive by RT-PCR. Thus, real-time PCR assay found to be sensitive and rapid for detection and quantification of JEV in pig blood samples. Attempts were made for isolation of JEV from two positive blood samples (positive by both RT-PCR and Real-time PCR) were subjected for isolation of JEV using Vero cell line, but JE virus could not be isolated from any of the sample. Out of 426 serum samples 101 (23.7%) were found positive by IgG targeted ELISA. Of the 165 sera samples collected from Piggery farm, IVRI, pork retail markets of Bareilly, Gorakhpur and Balia districts of Uttar Pradesh, 48 (29.09%) samples found positive for IgG antibodies against JE. Out of 261 samples collected from Deonar slaughter house, Mumbai, 53 (20.30%) samples found positive for JE. The NS3 gene sequences of GKP1_India_IVRI_VPH and GKP2_India_IVRI_VPH exhibited maximum nucleotide sequence identity of 100% with Japan JEV strain JaOArs982 (M18370), 98.8% similarity with mosquito isolate of JEV from China (JN 864064) and SA-14-14-2 strain of China (KC517497). It shared about 95.7% identity with two human isolates of JEV from West Bengal, India (JX050179 and JX072965). Thus, the present investigation clearly reveals the presence of JEV in pigs through application of RT-PCR, Real-time RT-PCR and indirect ELISA.

लघु सारांश



8 लघु सारांश

प्रस्तुत अन्वेषण जापानी मस्तिष्क ज्वर की शूकरों त्वरित जाँच हेतु व्युत्क्रम ट्रॉंसक्रिप्टेज–पीसीआर (आरटी–पीसीआर) व रियल टाइम पीसीआर आमापन को मानकीकरण करने के उद्देश्य से किया गया तथा अपरोक्ष एन्जाइम सम्बद्ध इम्यूनोसॉरबेन्ट आमापन द्वारा शूकरों में उपस्थिति का अध्ययन, आरटी–पीसीआर रियल टाइम पीसीआर किया गया। आरटी–पीसीआर शूकरों के रक्त नमूनों में ''ई'' जीन के आवरण को लक्ष्यीकृत करके जापानी मस्तिष्क ज्वर की त्वरित जाँच हेतु किया गया। त्वरित जाँच के साथ जापानी मस्तिष्क शोथ ग्रसित शूकर रक्त नमूनों में परिमाणीकरण परिमाणात्मक रियल टाइम पीसीआर आमापन जेई वायरस के एनएस3 जीन को लक्षीकृत कर प्राइमरों का उपयोग कर किया जिसमें 162 बेस युग्म का प्रवर्धन उत्पाद प्राप्त हुआ। शूकर रक्त के कुल 426 नमूनों में से, 17(3.99%) जेई वायरस आरएनए, के लिए घनात्मक थे, क्योंकि सीडीएनए इन नमूनों में प्रवर्धन करने पर पीसीआर द्वारा प्रत्याशित 474 वेस युग्म का उत्पपाद प्राप्त हुआ। सभी आरटी–पीसीआर घनामत्मक नमूने उत्तर प्रदेश से एकत्र किये गये थे देओनार वधशाला, मुंबई से एकत्र नमूनों में कोई भी आरटी–पीसीआर द्वारा घनात्मक नहीं मिला। कुल 426 शूकर रक्त नमूने क्यू आर–पीसीआर आमापन द्वारा जाँचे परखे गये। जेई वायरस 18 (4.22%) शूकर रक्त नमूनों में पहचाने गये व परिमाणीकृत किये। प्रत्येक क्रिया में विषाणु की संख्या 2.52×10^2 कापी संख्या से 4.243×10^9 के मध्य पायी गयी। पूर्व में आरटी–पीसीआर द्वारा जाँचे गये घनात्मक नमूनों में रियल टाइम पीसीआर आमापन द्वारा 17 शूकर नमूनों में वायरस की पहचान हो पाई। इस प्रकार रियल–टाइम पीसीआर आमापन शूकर रक्त नमूने के जाँच व परिमाणीकरण के लिए सेंवदनशील व त्वरित तरीका पाया गया। दो घनात्मक नमूनों से जेई वायरस के आइसोलेशन हेतु प्रयास किये गयेजो रक्त नमूने (दोनों विधियों आरटी–पीसीआर व रियल टाइम पीसीआर द्वारा घनात्मक) वीरो–कोशिका पंक्ति का प्रयोग कर आइसोलेशन का प्रयत्न किया परन्तु किसी भी नमूने से आइसोलेशन संभव नहीं हो सका। सीरमीय 426 नमूनों में से 101 (23.7%) आईजी जी लक्षीकृत एलिसा विधि द्वारा घनात्मक पाये गये। 165 सीरमीय नमूने जोकि शूकर फार्म आईवीआरआई, बरेली व फुटकर बाजार, बरेली, गोरखपुर व बलिया जनपदों उत्तर प्रदेश से एकत्र किये गये। जेई विरूद्ध आइजीजी एन्टबाडीज द्वारा 48(29.09%) नमूने घनात्मक थे। देओनार पशु वध शाला, मुंबई के 261 एकत्रित नमूनों में से 53(20.30%) नमूने जेई के लिए घनात्मक पाये गये। जीकेपी1 भारत आईवीआरआई वीपीएच व जीकेपी2 भारत आईवीआरआई वीपीएच के एनएस3 जीन अनुक्रमों की जापान के जेईवी स्ट्रेन जेएओएआरएस982 (एम1 8370) के साथ 100% समानता दर्शायी, मच्छर से आइसोलेट किये गये चीन के जेई वायरस (जेएन 864064) व एसए–14–14–2 स्ट्रेन (केसी 517497) के साथ 98.8% समानता दर्शायी। पश्चिम बंगाल भारत के दो मानव आइसोलेटों (जेएक्स 050179) व (जेएक्स 072965) के साथ 95.7% समरूपता दर्शायी। इस प्रकार प्रस्तुत अन्वेषण आरटी–पीसीआर, रिअलटाइम–आरटीपीसीआर व अपरोक्ष एलिसा विधि का प्रयोग कर यह स्पष्ट रूप से जेई वायरस की शूकरों में उपस्थिति को दर्शाता है।

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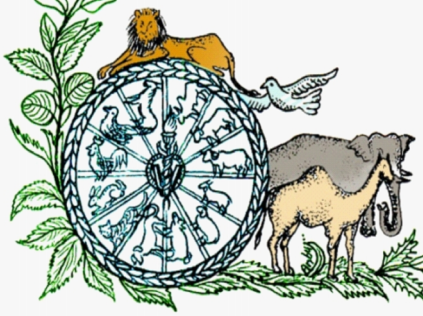

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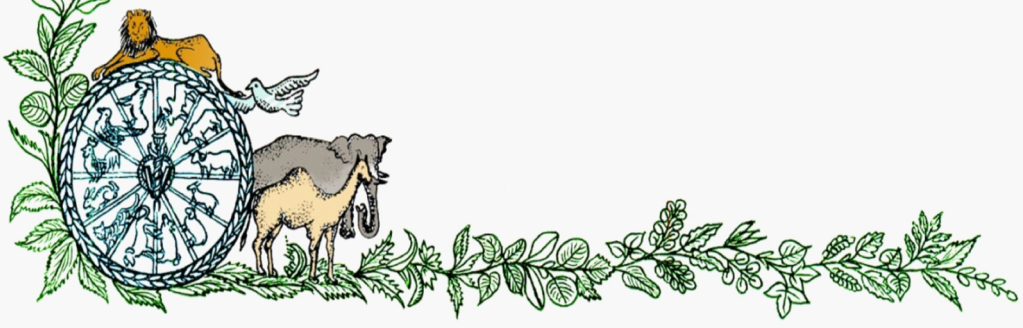
Appendix



APPENDIX

REAGENTS FOR MOLECULAR BIOLOGY

Ethidium bromide (10 mg/ml)

Ethidium bromide	10 mg
Distilled water	1 ml

The solution was stored in dark color bottle at 4°C.

TAE (5X)

Tris base	242 g
Glacial acetic acid	57.1 ml
EDTA (0.5 M)	100 ml

Adjust the pH to 8 and make volume to 1000 ml with distilled water.

REAGENTS FOR CLONING

Calcium chloride (0.1 M)

Dissolve 1.47g of Calcium chloride in 100 ml of distilled water and store at 4°C.

Ampicillin (100 mg/ml)

Dissolve 100 mg Ampicillin in 1 ml of sterile water or 70% ethanol. Mix well and filter using 0.22 µ filter and store at -20°C.

REAGENTS FOR PLASMID EXTRACTION

Solution PI/Resuspension buffer

Tris HCl (pH 8)	50mM
EDTA	10 mM

Adjust pH with NaOH and store at 4°C. Add RNase A 100 µg/ml at the time of use.

Solution PII/Lysis buffer

1 N NaOH	200 µl
10% SDS	100 µl
Autoclaved distilled water	700 µl

Make fresh at the time of use.

Solution PIII/Neutralization buffer

Potassium acetate (5 M)	60 ml
Glacial Acetic acid	11.5 ml

Adjust pH to 5.5 and make volume to 100 ml with distilled water and stored at 4°C.

Reagents for ELISA

Phosphate Buffered Saline 1X (pH 7.2)

Sodium chloride	8.00 gm
Potassium chloride	0.02 gm
Disodium hydrogen phosphate (anhydrous)	1.44 gm
Potassium dihydrogen phosphate	0.20 gm
Distilled water	1000 ml

Sterilized by autoclaving and stored at 4°C until used.

Washing buffer (PBS-T)

PBS (pH 7.4)	100 ml
Tween 20	50 µl

Coating buffer

Sodium bicarbonate (35 mM)	2.9 gm
Distilled water	1000 ml

Adjust the pH to 9.5 and store at 4°C.

Blocking buffer

Skimmed milk powder	5 gm
Washing buffer (PBS-T)	100 ml

ELISA substrate

1. Citrate buffer 0.1 M (pH 4.6)

Citric acid	2.1 gm
Sodium citrate	2.94gm
Deionized water	100 ml

Adjust pH to 4.6 and store at 4°C

2. Substrate solution

Citrate buffer (pH 4.6)	10 ml
H_2O_2 (30%)	8 µl
O-phenylene diamine dihydrochloride (OPD)	10 mg

Make fresh at the time of use.

VITAE

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