Chapter - 3

MATERIALS AND METHODS

3.1: Chikungunya epidemic

Andhra Pradesh was the first state (from India) to report CHIKV infection during December 2005 and one of the worst affected (Ravi, 2006). During 2008-2009, we observed a huge number of patients in kadapa district of Andhra Pradesh with crippling arthralgia and fever. According to the CHIKV case definition by National Institute of Communicable Diseases (NICD), New Delhi, CHIKV infection was suspected in these patients.

3.2: Surveillance and Seroepidemiology

3.2.1: Survey

A survey was carried out from July 2008 – March 2009 in Chikungunya epidemic suspected villages of Kadapa district, Andhra Pradesh. Prior to sample collection "Informed oral Consent" were obtained from all the patients and their parents (in case of minors).

3.2.2: Questionnaire preparation

A CHIK questionnaire was prepared with the assistance of District Malaria Officer (DMO), Kadapa district. The first half of the questionnaire enquired about the patient's name, age, gender, contact address, telephone number. The second part of the questionnaire contained multiple choice questions to be answered by ticking the appropriate boxes and focused on the clinical signs and symptoms observed in the patients. The duration of each symptom was noted. Details of mosquito breeding sites, preventive measures adopted and medication used by the patients were also noted. A sample questionnaire form is shown in Annexure 1.

3.2.3. Clinical Symptoms

The most common symptoms of the CHIK were found to be fever for 2-3 days, severe arthralgia, myalgia and swelling of tender joints like wrist, palms, ankles, rash etc., The information gathered regarding clinically diagnosed symptoms were analyzed.

3.3: Sample collection

Approximately 2-3 ml blood samples from CHIK suspected patients were collected in sterile screw capped vials of 3ml capacity. The samples were drawn form the patients under the observation of Mobile Medical Unit, Kadapa district of Andhra Pradesh. Then the samples were transported under cold conditions to the laboratory and serum was separated within 24 hrs. Acute sera were stored at -80˚C until assayed for virus detection by RT – PCR, whereas the convalescent sera were stored at -20˚C until assayed for antibodies against CHIK.

3.3.1: Sample analysis

Convalescent phase samples were screened for the presence of anti-chic IgM antibodies using Rapid Immunochromatographic assay (RICA) or one step IgM antibodies to CHIKV and IgM Antibody Capture – Enzyme Linked Immuno Sorbet Assay (MAC-ELISA). Acute phase samples were screened for the presence of CHIKV specific RNA by using the RT-PCR assay.

3.3.2: Rapid Immunochromatographic assay (RICA)

Fig. 3.1a): SD BIOLINE Chikungunya IgM test Kit (Source: Standard diagnostics, Inc., South Korea)

RICA (SD Beeline, South Koran) is a solid phase immunochromatographic assay for rapid, qualitative detection of IgM antibodies to CHIKV in human serum or plasma. The assay can be performed without any requirement for special equipments or technical skills and the result is readily available within 10 min.

Serum samples were assayed for the presence of Anti-Chik IgM according to manufacturer's instructions.

- The kit components (Test device, Assay diluent) and Specimen (Serum) were brought to room temperature prior to testing.
- The test device was placed on a flat surface and one drop (about 50 µl) of serum was loaded into the sample well of the test device.
- One drop of assay diluent was added into the sample well and allowed to pass through the membrane.
- The result was interpreted after 10 minutes.

Interpretation of the test result (Fig 3.1) (source: Standard diagnostics Inc., South Korea)

- Negative result: The presence of only one purple band (“C”) within the result window indicates a negative result.
- Positive result: The presence of two purple bands (“T” and “C”) within the result window indicates a positive result.
- Invalid result: If the ourole colour band is not visible or only test band (“T”) is visible within the result window, the result is considered invalid and the specimen was retested.

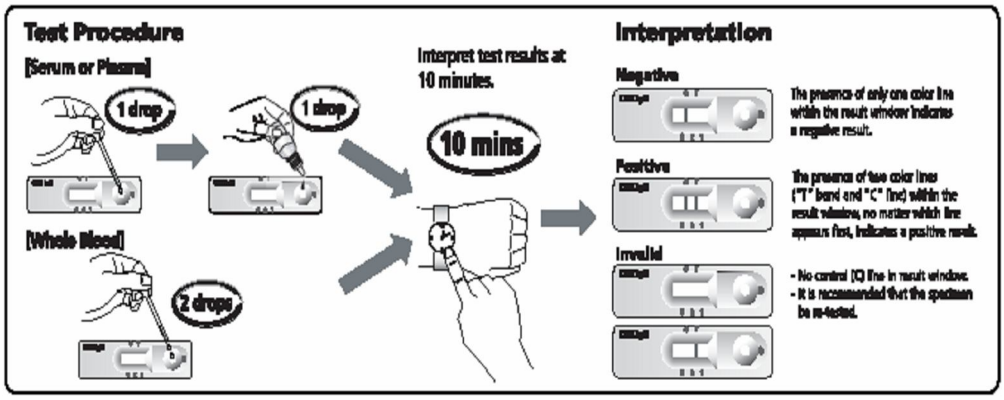

Fig. 3.1b): **Diagram depicting the assay procedure for qualitative detection of IgM antibodies to CHIKV and interpretation of results (Source: Standard diagnostics, Inc., South Korea)**

3.3.3: IgM Capture Antibody – Enzyme Linked Immunosorbant Assay (MAC-ELISA)

RICA anti-Chik IgM positive sera samples were assayed for the presence of IgM antibodies against CHIKV by using MAC-ELISA (NIV, Pune) according to manufacturer’s instructions.

Ninety - six well microtiter plate pre coated with anti-human IgM antibody was washed, sera for testing were diluted 1:1 in serum dilution buffer and 50 µl added to the wells in replicates with two wells serving as positive control and two wells serving as negative control to determine the cutoff value. Initial testing of

the serum samples was done at 1:100 dilutions as per manufacturer's recommendations. Followed by one hour incubation at 37^0C, washed and 50 µl CHIKV antigen was added to each well. After one hour incubation at 37^0C and washing 50 µl of CHIKV antigen specific monoclonal antibody was added to each well. Then after incubation at 37^0C/hour and washing, 50 µl of Avidin – Horse Radish Peroxidase Enzyme conjugate was added and incubated for additional half –an hour at 37^0C and washed the plate. Then Anti-human IgM antibody bound CHIKV IgM antibody- CHIKV- antigen – CHIKV- Ag specific antibody sandwiched with conjugate was detected by adding 50 µl 1:20 diluted TMB substrate. The substrate was allowed to react for 10 minutes in dark at room temperature. Then the reaction was terminated by adding 100 µl 1N H_2SO_4. The plate was read at 450nm in ELISA Reader (Model 680 Microplate Reader S/N 19548). The optical density of each serum was determined by subtracting its OD obtained from the negative control OD. The sample was considered positive when its OD is the same or more than the positive control OD obtained and subtracted from negative control OD.

3.3.4: Comparative sensitivity of RICA and MAC-ELISA

The sera samples for anti-Chik IgM in RICA further assayed using MAC-ELISA but only few samples were found positive by the development of colour. The result of analyzed sera using both assays was summarized. The sensitivity of RICA and MAC-ELISA was determined.

3.4: Molecular diagnosis of Chikungunya

3.4.1: Preparation of RNase-free materials

All the plasticware necessary for the isolation of RNA and RT-PCR, like eppendorf tubes (1.5ml), PCR tubes(0.2 ml) and tips (1ml, 200 µl, 10 µl) were soaked in 0.01% DEPC treated water (100 µl of DEPC in 1 L of distilled water and stirred overnight). The DEPC treated plasticware were dried and autoclaved for further use.

3.4.2: Extraction of RNA from CHIKV infected patient sera

Genomic viral RNA was extracted from CHIKV infected patient serum by using TRIZOL, LS reagent according to the manufacturer's instructions (Invitrogen, CA, USA). Addition of chloroform followed by centrifugation separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase, which is recovered by precipitation with chilled isopropyl alcohol.

Procedure

250 µl of serum sample was mixed with 750 µl of TRIZOL LS reagent in proportion of 1:3 and incubated at room temperature for 15 min. To the lysate 200 µl of chloroform was added and the solution was mixed by inversion for 15 sec. The mixture was allowed to stand for 5 min at room temperature. Then the mixture was centrifuged at 13,000 rpm for 10 min at 4°C and the aqueous phase (~500 µl) at the top was transferred to a sterile microfuge tube of 1.5 ml capacity. To the aqueous phase chilled Isopropyl alcohol (500 µl) was added in 1:1 proportion to precipitate the RNA present in the aqueous phase, mixed properly and incubated at room temperature for 30 min. The tubes were centrifuged at

13,000 rpm for 15 min at 4°C and the supernatant was discarded and the RNA pellet was washed with 500 µl of chilled 70% alcohol by centrifuging at 10,000 rpm for 5 min at 4°C. Then the pellet was air-dried to remove ethanol and reconstituted in 30 µl of RNase-free DEPC treated water. The extracted RNA was stored at -20°C for further use.

3.4.3: Reverse Transcription or Complementary DNA (cDNA) synthesis

cDNA was synthesized from extracted RNA by using RevrtAid M-MuLV Reverse Transcriptase (Fermentas, USA) and Oligo $(dT)_{18}$ primer. Reverse transcription mix was prepared to a final volume of 20 µl in a 0.2 ml sterile PCR tube. The composition of reverse transcription mix for one reaction is as follows:

Reaction mixture

5X RT Buffer	4.0 µl
10mM dNTPs Mix (1mM final conc.)	2.0 µl (1mM final conc.)
100 µM Oligo $(dT)_{18}$ Primer	1.0 µl (0.5 µg)
Riboblock RNase Inhibitor (40U/ µl)	0.5 µl (20U)
RevertAid M-MuLV RT enzyme (20U/ µl)	1.0 µl (20U)
Total RNA (template)	3.0 µl
DEPC treated water	8.5 µl
Total volume	**20.0 µl**

The reaction mix was incubated at 42°C for 60 min and the reaction was terminated by heating the reaction mix at 70°C for 10 min. Then the synthesized cDNA was stored at -20°C for further use.

3.4.4: Primer design

CHIKV re-emerged in December, 2005 in India (Ravi, 2006), by that time, there were no documented reports identifying the circulating CHIKV genotype in India. An earlier study indicated that the three CHIKV genotypes (ECSA, Asian and West African) were restricted to their geographical areas as indicated by their names (Powers *et al.*, 2000). Hence based on the above hypothesis, we designed a primer pair DVRChik-F/DVRChikR (Table 3.1 and Fig 3.2) which could amplify a 330bp product specific to CHIKV E1 gene. Primers were designed based on CHIKV strain 653496 (Accession No: AY424803) from an epidemic at Nagpur in 1965. Oligonucleotide primers were synthesized from Eurofins Genomics India Pvt. Ltd. (Formerly known as MWG Biotech Pvt. Ltd., Bangalore). Working solutions of the primers (10 pmol/µl) were prepared from original stock (100 pmol/ µl) by using RNase free DEPC treated water.

Table 3.1: The sequence and position of the diagnostic primers

Primer	**Sequence (5' to 3' direction)**	**Orientation**	**Genome Position* (bp)**	**Product size**
DVRChik-F (Forward Primer)	ACCGGCGTCTACCCATTCATGT	Sense	10237 to 10258	330bp
DVRChik-R (Reverse Primer)	GGGCGGGTAGTCCATGTTGTAGA	Antisense	10544 to 10566	

***Genome position is according to the Chikungunya virus strain S27-African prototype (AF369024).**

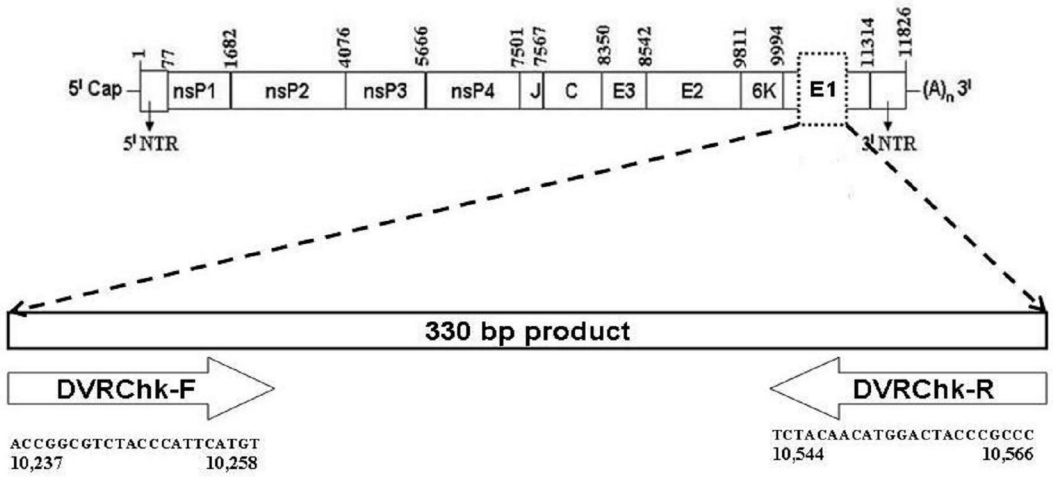

Fig.3.2: Location of primer binding sites with respect to *Chikungunya virus* strain S27-African prototype (AF369024) DVRChk-F: Forward primer; DVRChk-R: Reverse primer.

3.4.5: Standardization of RT-PCR

PCR conditions were standardized by varying the annealing temperatures, $MgCl_2$ concentration and number of amplification cycles (repeats). 3 µl of cDNA was amplified in a 20 µl reaction volume using thermal cycler (Corbett Research, Model CGI-96, Australia). The composition of PCR mix is as follows:

PCR Mix

Reagents	**Volume**
10X Taq Buffer	2.0 µl
25mM $MgCl_2$	1.6 µl (2mM)
10mM dNTP mix	0.4 µl (0.2 mM final conc.)
DVRChik-F (Forward Primer)	1.0 µl (10 pmol)
DVRChik-R (Reverse Primer)	1.0 µl (10 pmol)
Taq DNA Polymerase (5U/ µl)	0.5 µl (2.5U)
Template (cDNA)	3.0 µl
Sterile double distilled water	10.5 µl
Total volume	**20.0 µl**

The PCR conditions were as follows:

Step No.	Temperature	Time	No. of cycles	Remarks
1.	94° C	5 min	1	Initial denaturation
2.	94°C	45 sec	35	Denaturation
	56°C	30 sec		Annealing
	72°C	1 min		Extension
3.	72°C	30 min	1	Final extension
4.	4°C	Soak	-	Hold

The final extension step ensured the highest efficiency of 3' –dA tailing of PCR products which is necessary for subsequent cloning into pGEM-T Easy vector (T/A vector).

3.4.6: Confirmation of RT-PCR products in 1% Agarose Gel Electrophoresis

The amplified PCR products (330bp) were analyzed by 1% agarose gel electrophoresis (Sambrook and Russel, 2001). 500 mg of agarose was dissolved in 50ml of 1X TBE buffer (Appendix) using a micro oven. Ethidium bromide (1 µg/ml agarose) was added to molten agarose after cooling and then poured into a horizontal agarose gel boat with comb. The PCR amplified products mixed with 5X DNA loading dye and GeneRuler™ Ladder (Fermentas, Canada) were separately loaded into the wells of the gel and electrophoresed by keeping the gel boat in electrophoresis tank containing 1X TBE buffer at 100 V until the dye front reached almost the other end of the gel. The migration pattern of the DNA fragments in the gel was recorded using Gel Documentation System (Alpha Innotech, USA).

3.4.7: Gel purification of PCR Product

The PCR products were Gel extracted using Manufacturer's (QIAquick Gel Extraction Kit Cat # QIAGEN Inc. USA) protocol.

1 % LMP Agarose gel was casted and 50 µl PCR product was mixed with 6x gel loading dye and electrophoresed at 60 volt for 1.5 hours as described previously. After electrophoresis, the gel was briefly visualized and the desired bands were excised using a sterile sharp scalpel and the gel slice was transferred to a pre-weighed 1.5 ml microfuge tubes. The gel slice weight was measured and added with 3 volumes of Buffer QG to a 1 volume of gel slice (100mg ~ 100 µl) and incubated at 50°C for 10 min. with intermittent vortexing every 2-3 min. After the gel slice completely dissolved, isopropanol equivalent to gel volume was added and mixed. QIAquick spin column was placed in provided 2 ml collection tube. For binding the DNA, the sample was applied to the QIAquick spin column and centrifuged at 12,000 g for 1 min. The flow-through was discarded, the QIAquick spin column again placed into the same tube. 500 µl of Buffer QG was added to QIAquick spin column and centrifuged for another 1 min at 12000 g. For washing, 750 µl of Buffer PE was added to the QIAquick spin column and centrifuged at 12000 g for 1 min after incubating at room temperature for 5 min. The flow-through was discarded and QIAquick spin column again placed into the same tube, centrifuged for an additional 2 min at 12000 rpm. Then QIAquick spin column was placed in a sterile 1.5ml microcentrifuge tube. For final elution, 20 µl of Buffer EB was added to the centre of the column and centrifuged for 2 min at 12000 rpm after incubating the column for 2 min. The purified PCR product was checked by running 2 µl of the same in 1 % agarose gel.

3.5: Cloning of E1 gene amplicon of CHIKV

The gel purified PCR products were cloned into pGEM-T Easy vector system (Promega, USA)(Fig 3.3) according to the manufacturer's protocol.

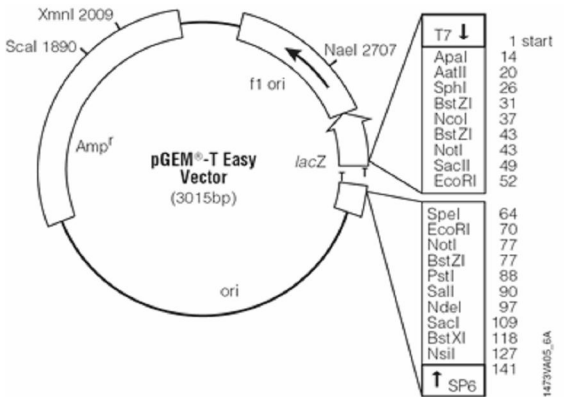

Fig. 3.3: Genetic map of pGEM-T Easy vector (Source: Promega, USA)

3.5.1: Ligation of the PCR products

The pGEM-T Easy vector tube was briefly spun to collect the contents at the bottom. The 2X Rapid Ligation buffer was vortexed vigorously.

Following reagents were added in a 0.5 µl reaction tube.

S.No	Reagents	Volume
1.	2X Rapid Ligation buffer	5.0 µl
2.	pGEM-T Easy vector	1.0 µl
3.	Purified PCR product	2.0 µl
4.	T4 DNA ligase	1.0 µl
5.	Nuclease free water	1.0 µl

The reaction mixture was mixed by pipetting. Then it was incubated at room temperature for 1 hour and transferred to 4°C overnight for maximum number of transformants. Then the ligated product was stored at -20°C until use.

3.5.2: Preparation of Competent E.Coli cells (Sambrook and Russel, 2001)

Escherichia coli DH5α glycerol stock was taken with an inoculation loop and streaked onto LB agar (HiMedia, Mumbai) (Appendix) plates without antibiotics and incubated at 37°C for overnight. A single colony was picked up from the culture plate and sub cultured into 10ml of LB broth (HiMedia, Mumbai) and incubated overnight at 37°C with constant shaking at 150 rpm using incubator-shaker (Innova 4080, New Brunswick Scientific). 1ml of overnight culture was inoculated in 100ml LB broth (without antibiotic) and grown till the OD_{600} reached to 0.4-0.5. The culture was chilled on ice for 20 min and transferred the chilled culture to pre-chilled sterile 1.5 ml microfuge tubes (1.5ml culture/tube). Then the tubes were chilled on ice for 10 min and the cells were spun down at 5000 rpm for 5 min at 4°C. The supernatant was discarded and the cell pellet was resuspended in 750 µl of ice cold filter sterilized 100 mM $CaCl_2$. The tubes were then incubated on ice for 15 min. The cells were again centrifuged and resuspended gently in 200 µl chilled 100 mM $CaCl_2$. The cells were either used after overnight incubation on ice (or) frozen in liquid nitrogen and stored at -80°C.

3.5.3: Transformation

The frozen competent cells were thawed on ice for 10 min. 10 µl of the above ligation mixture was mixed by swirling gently with competent cells in 1.5ml microcentrifuge tube and incubated on ice for 30 min. The cells were given a heat shock at 42°C for 90 sec and rapidly chilled on an ice-bath for 5 min. Then the cells were added to 1 ml LB medium and incubated with vigorous shaking at 37°C for 60 min. The cells (100-200 µl each) were then plated onto LB agar

plates containing ampicillin (50 µg/ml), X-Gal and IPTG (Appendix). The plates were incubated overnight at 37°C for the development of colonies (Sambrook and Russel, 2001).

3.5.4: Selection of Recombinant clones

The recombinant clones were selected by blue-white colony screening system because of the TA vector used. The recombinant colonies showing white colour due to the insertional inactivation of the lacZ gene were selected for further screening.

3.5.5: Isolation of plasmid DNA (Miniprep)

The white colonies were picked with a toothpick and inoculated separately into 3 ml of LB broth containing ampicillin and incubated overnight at 37°C at 200 rpm shaking. The culture was transferred to microcentrifuge tube and pelleted at 5000 rpm for 5 min at 4°C. The pellet was resuspended in 300 µl of cold solution I (Appendix) by vigorous vortexing. 300 µl of solution II (Appendix) was added, mixed by inverting tubes 4-5 times and incubated at room temperature for 5 min. Subsequently 300 µl of solution III (Appendix) was added and mixed by inverting the tubes. Then the tubes were incubated on ice for 5 min and centrifuged at 14,000 rpm for 10 min at room temperature. The supernatants were transferred to a fresh microcentrifuge tubes and 600 µl of chilled isopropanol was added, mixed and centrifuged at 14,000 rpm for 30 min. The pellet was washed twice with chilled 70% ethanol (500 µl each time) and air dried (usually 5 to 10 min at 37°C). Then the pellet was dissolved in required amount of sterile

distilled water. Selected colonies were compared by the mobility of the recombinant plasmids with respect to the vector DNA.

3.5.6: Confirmation of recombinant clones by colony PCR

Recombinant plasmids were used as a template for PCR analysis using DVRChik-F/DVRChik-R primers (Diagnostic Primers) as mentioned in section 3.4.5. The amplified products were checked by 1% agarose gel electrophoresis as described in section 3.4.6

3.5.7: Confirmation of recombinant clones by Restriction enzyme digestion

Recombinant plasmids were further screened by restriction digestion with *Eco*RI (New England Biolabs Inc., USA) according to manufacturer's instructions. The Restriction digestion mixture was prepared as follows:

10X *Eco*RI buffer	2.0µl
Restriction enzyme (*Eco*RI)	1.0 µl
Sterile distilled water	2.0 µl
Plasmid DNA	5.0 µl
Final volume of reaction mix	**10.0 µl**

The restriction enzyme mix was incubated at 37°C for 1 hour and the digested products were agarose gel electrophoresed as mentioned section 3.4.6. The potential recombinants with expected size DNA insert (330bp) were sequenced using T7 and SP6 universal primers at Eurofins Genomics India Pvt.Ltd., Bangalore, India.

3.5.8: Sequence analysis of E1 gene amplicon (330bp)

The sequenced contigs were manually assembled, screened for the vector sequence and low quality data using Chromas Lite tool. The vector sequence and low quality data were trimmed and the contigs were assembled using CAP assembly program of BioEdit package V.5.0.9. The partial nucleotide and deduced amino acid sequences of E1 gene obtained in the present study were compared with the reported CHIKV sequences from different geographical regions (Table 3.2) using Mega version 4.0. (Tamura *et al*., 2007). Sequence alignments and Phylogenetic analysis was performed using Clustal X Package V. 1.83. The phylogenetic trees were constructed at nucleotide level and aminoacid level and visualized using Tree Explorer V.2.12.

Table 3.2: Source of E1 gene sequences of *Chikungunya virus* isolates used in this study for Comparison

Sl.No.	Genbank Accession number	Strain/Isolate	Country/Year	Reference
1.	AF369024	S27-African prototype	Japan - 1953	Khan *et al*., 2002
2.	EF027139	IND-00-MH4	India - 2000	Arankalle *et al*., 2007
3.	HQ456255	Lamu33	USA - 2004/05	Kariuki Njenga *et al*., 2008
4.	HQ456253	Com125	USA – 2004/05	Kariuki Njenga *et al*., 2008
5.	AM258990	05-115	Reunion – 2005	Schuffenecker *et al*., 2006
6.	AM258993	06-027	Reunion – 2005	Schuffenecker *et al*., 2006
7.	GQ428210	RGCB03/KL06	India – 2007	Sreekumar *et al*., 2010
8.	FJ000068	IND-KA51	Karnataka, India – 2006	Cherian *et al*., 2009
9.	EF027138	IND-06-TN1	India – 2005/06	Arankalle *et al*., 2007
10.	FJ000065	IND-GJ53	Gujarat, India - 2006	Cherian *et al*., 2009
11.	EF027137	IND-06-RJ1	India - 2007	Arankalle *et al*., 2007
12.	FJ000067	IND-MH51	Maharashtra, India – 2006	Cherian *et al*., 2009
13.	EF027134	IND-06-AP3	India – 2006	Arankalle *et al*., 2007
14.	EF210157	DRDE-06	India – 2006	Santhosh *et al*., 2008
15.	GQ428212	RGCB80/KL07	India – 2007	Sreekumar *et al*., 2010
16.	EU886198	CHIK-AP-NLR	AP, India – 2006	unpublished
17.	KF587904	CHIK-Kadapa UHC	AP, India – 2008	Sangamithra et al., (Present Study)
18.	KF5879505	CHIK- Devapatla	AP, India – 2009	Sangamithra et al., (Present Study)
19.	HQ529778	CHIK-E1SVU07	AP, India – 2007	unpublished
20.	JN558835	SVUKDP-08	AP, India - 2008	unpublished
21.	FJ705369	CHIK-Sannapalle	AP, India – 2008	unpublished
22.	FJ705370	CHIK-Thudumaladinne-01	AP, India – 2008	unpublished
23.	GQ229487	CHIK-Dadireddypalli	AP, India – 2008	unpublished
24.	GQ229486	CHIK-Kamalapuram	AP, India – 2009	unpublished
25.	GQ229488	CHIK-Peddaputha01	AP, India – 2009	unpublished
26.	JN558834	SVUCTR-09	AP, India – 2009	unpublished
27.	JN558836	SVUKDP-09	AP, India – 2009	Unpublished