FR 1682

FINAL REPORT

Development of recombinant proteins as diagnostic intermediates for Chikungunya (CHIK), dengue and leptospirosis infections

Grant number (RG/2009/BT/01)

Dr. Menaka Hapugoda

Molecular Medicine Unit

Faculty of Medicine

University of Kelaniya

Ragama

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iii. ACKNOWLEDGEMENTS

It is with deep affection, appreciation and gratitude that I record here, my sincere thanks to the National Science Foundation (NSF) for granting me this student assistant ship project (Grant no. RG/2009/BT/01) in honour of a Collaborative Research Project granted by the International Centre for Genetic Engineering and Biotechnology (ICGEB) (ICGEB-SRI08-02). I express my sincere gratitude and sincere thanks to the Director, NSF. I owe a special debt of gratitude to Dr. S. A. V. Moorthy Senior Scientific Officer for her kind co-operation and invaluable advices during past few years.

I acknowledge to Miss. Maheshi Athapaththu, Research Assistant involved in the project her untiring effort extended through out the project. I would like to thank to Miss. Thanuja Denipitiya for covering up Miss. Athapaththu's activities when she left the project for writing her Ph.D. thesis.

I would like to express my deep appreciation to the ICGEB and International Atomic Energy Agency (IAEA TC 5/042 project) for facilitating Miss. Athapaththu, Research Assistant with fellowship trainings.

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My deeply grateful acknowledgement goes to patients and volunteers who voluntary provided clinical samples without hesitation.

Finally, I would like to express my deep appreciation to all academic and technical staff members of the Molecular Medicine Unit, Faculty of Medicine, University of Kelaniya, Sri Lanka for helping me through out the project period.

SECTION 1.

INFORMATION REGARDING PROJECT/PROJECT PERSONNEL

i. **Grant number**

RG/2009/BT/01

ii. **Title of project**

Development of recombinant proteins as diagnostic intermediates for Chikungunya (CHIK), dengue and leptospirosis infections

iii. **Principle investigator (Prof/Dr/Mr/Ms)**

Dr. Menaka Hapugoda

iv. **Co- investigators**

No

v. **Institute where research was being carried out**

- Molecular Medicine Unit, Faculty of Medicine, University of Kelaniya, Ragama, Sri Lanka
- International Centre for Genetic Engineering and Biotechnology(ICGEB), New Delhi Resident, India
- Dept. Of Virology, WHO collaborative Centre for viral reference and research, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan

vi. **Date of award**

22.07.2009

Date project was initiated (if different from above): .6.07.2010

vii. **Date of completion of the project**

30/11/2013

viii. **Total allocation of funds (Rs): 1,087,000.00**

ix. **Total spent (Rs): 963,161.29**

x. **Number of research students employed**

Student 1. From 06.07.2010 to 19.03.2013

Name: Miss. Maheshi Athapaththu

Registered for degree: Ph.D. degree in Molecular Medicine, Faculty of Graduate Studies, University of Kelaniya (FGS/05/02/09/2009/01). She submitted her thesis on 13th of December, 2013 and *viva voice* examination was held on 3rd of July, 2014. The date of award of her Ph.D. degree was 13th of December, 2013.

Student 2. From 20.03.2013 to 20.11.13

Name of Research student: Miss. Thanuja Denipitiya

Registered for degree: Reading for a Ph.D. degree in Molecular Medicine, Faculty of Science, University of Colombo (2012/M.Phil/S/70).

Miss Maheshi (student 1) wanted to concentre writing of her thesis therefore, she has resigned from the post of research assistant before ending of the project. Only analysis of recombinant proteins prepared for leptospirosis was remaining when she left the project.

Miss. Thanuja (student 2) has analysed recombinant protein antigens prepared for leptospirosis using clinical samples and completed remaining project activities.

xi. Number of Technical Assistants / labourers employed and period of service

No

xii. Publications/communications arising from the project during the reporting period (Please attach copies): Please refer Annexure 4.

Conference proceedings –International

1. Athapaththu, A. M. M. H., Khanna, N., Abeyewickreme, W., Gunasena, S. and Hapugoda, M. (2010). Enzyme-Linked Immunosorbent Assay (ELISA) using recombinant protein antigens for detection of anti-chikungunya antibodies. *Proceedings of the Joint International Tropical Medicine Meeting*, Bangkok 103.

Conference proceedings -National

1. Athapaththu, A. M. M. H., Khanna, N., Gunasena, S., Abeyewickreme, W. and Hapugoda, M. (2010). Development of recombinant proteins as diagnostic intermediates for chikungunya infection. *Proceedings of the Sri Lanka Association for the Advancement of Science* **65:** 10.

2. Athapaththu, A. M. M. H., Khanna, N., Inouve, S., Tun, M. M. N., Gunasena, S., Abeyewickreme, W. and Hapugoda, M. (2011).

Comparison of recombinant protein and cell lysate antigens for detection of anti-chikunungya IgM antibody. *Proceedings of the Sri Lanka College of Microbiologists.* **09:** 16 *(This abstract has been awarded the second place in oral presentation by the College of Microbiologist).*

3. Athapaththu, A. M. M. H., Khanna, N., Inouve, S., Tun, M.M.N., Gunasena, S., Abeyewickreme, W. and Hapugoda, M. (2013). Development of recombinant protein antigens using a bacterial expression system for the detection of anti-Chikungunya (CHIK) antibodies. *Proceedings of the Sri Lanka College of Microbiologists.* **11 (1):** 14 *(This abstract has been awarded the second place in oral presentation by the College of Microbiologist).*

4. Athapaththu, A.M.M.H., Khanna, N., Inouve, S., Gunasena, S., Abeyewickreme W. and Hapugoda, M. (2013). Comparison of recombinant Chikungunya (CHIK) E2 antigens expressed in bacterial and eukaryotic systems for the detection of anti-CHIK antibodies in human serums samples. *Proceedings of the 14th of Annual Research Symposium, Faculty of Graduate Studies, University of Kelaniya* 68-69.

5. Athapaththu, A.M.M.H., Khanna, N., Inouve, S., Gunasena, S., Abeyewickreme W. and Hapugoda, M. (2013). Development of recombinant protein antigens using a yeast expression system, for the detection of anti-Chikungunya (CHIK) antibodies in clinical samples. *Proceeding of the Annual scientific sessions Sri Lanka Association for the Advanced of Science (SLAAS)* 12.

6. Denipitiya, D.T.H., Chandrasekharan, N.V., Abeyewickreme, W. and Hapugoda, M.D. (2013). Detection of pathogenic *Leptospira* in rat blood samples by molecular-based assays. *Proceedings of the 14th of Annual Research Symposium, Faculty of Graduate Studies, University of Kelaniya* 72-73.

7. Denipitiya, D.T.H., Athapaththu, M., Chandrasekharan, N.V., Abeyewickreme, W. and Hapugoda M.D.(2013). Risk factors associated with human leptospirosis in the District of Gampaha, Sri Lanka. *Proceedings of the 14th of Annual Research Symposium, Faculty of Graduate Studies, University of Kelaniya* 73-74.

8. Denipitiya, D.T.H., Jiffriy, A.M., Chandrasekharan, N.V., Abeyewickreme, W. and Hapugoda, M.D. (2013). Evaluation of a real time Polymerase Chain Reaction (PCR) assay for the early diagnosis of human leptospirosis. *Proceeding of the Annual scientific sessions Sri Lanka Association for the Advanced of Science (SLASS)* 21.

9. Denipitiya, D.T.H., Chandrasekharan, N.V., Abeyewickreme, W. and Hapugoda, M.D. (2013). Evaluation of a case definition for leptospirosis diagnosis using serological assays. *Proceedings of the Annual scientific sessions, Sri Lanka Association for the Advanced of Science (SLAAS)* 20.

10. Denipitiya, D.T.H., Jiffriy, A.M., Chandrasekharan, N.V., Abeyewickreme, W. and Hapugoda, M.D. (2014). A comparison of three molecular-based assays to detect pathogenic leptospires in cattle urine. *Proceedings of Peradeniya University International Research Session* **18:** 378.

Research papers-Under preparation

SECTION 2.

EXECUTIVE SUMMARY OF THE PROJECT

Scientific background: Chikungunya (CHIK), dengue and leptospirosis are important diseases with explosive outbreaks occurring in Sri Lanka. Confirmation of these outbreaks is important for clinicians for proper management of patients but it is difficult to confirm diagnosis of these three diseases only on clinical grounds as some of their clinical symptoms overlap with each other. Enzyme-Linked Immunosorbent Assay (ELISA) can be used for confirmation of these diseases. Use of ELISAs in Sri Lanka is hindered by the high cost of commercial diagnostic kits and inaccessibility of reagents. Further, whole viral/bacterial antigens/lysate used in ELISAs can cause biohazard risk, high production cost and cross reactivity with other organisms of the same genus/family. Therefore, a diagnostic intermediate for ELISA produced at low cost and easily standardized for use in field settings is important.

Objective: To assist confirmation of CHIK, dengue and leptospirosis outbreaks through developing rapid laboratory diagnostic assays.

Methodology: Novel recombinant protein antigens for all three diseases were prepared. Indirect ELISAs using each novel recombinant protein as a capture antigen for detection of both anti-IgM and IgG antibodies of each disease were developed. Potential use of each protein as a diagnostic tool for the detection of both IgM and IgG antibodies produced against the particular disease organism was evaluated with currently available diagnostic assays including the Gold standard assay using a large panels of well characterized serum samples.

Major findings: Single recombinant protein antigen to detect both IgM and IgG antibodies of each disease was developed and evaluated.

SECTION 3.

REPORT IN DETAIL

i. Introduction/Background

Chikungunya, dengue and leptospirosis are important diseases causing severe outbreaks in the recent past in South East Asian region including Sri Lanka. Chikungunya, dengue and leptospirosis are diseases caused by alphaviruses, flaviviruses and pathogenic spirochetes bacteria respectively. During outbreaks, confusion between the broad spectrum of clinical presentations associated with these three diseases complicate the early diagnosis required for the timely patient management. Confirmation of disease outbreaks is important for clinicians for proper management of patients and control programmers for surveillance. Currently available laboratory diagnostic assays (Table 1) depend on availability of disease organisms in human and human immunological responses to a selected disease organism (Figure 1).

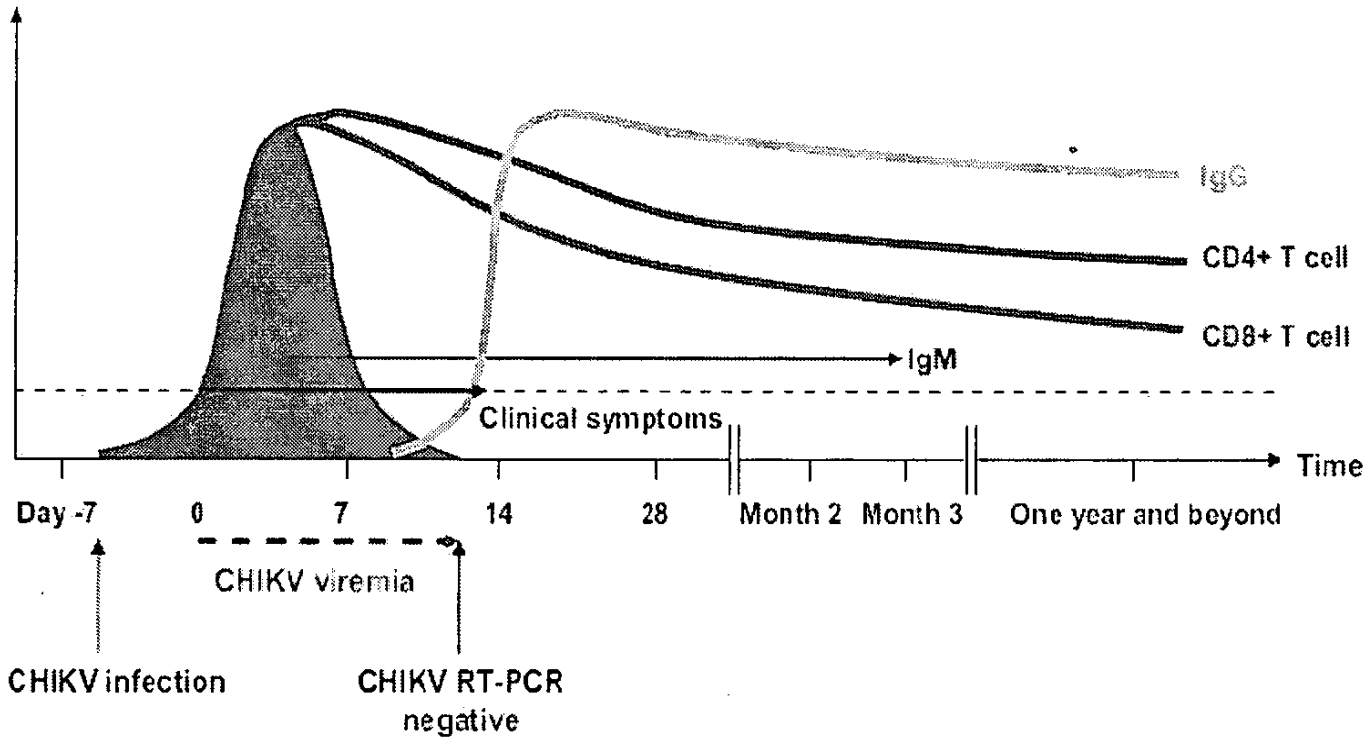


Figure 1. Human immunological responses to a disease organism

Table 1. Currently available laboratory diagnostic assays for CHIK, dengue and leptospirosis

Fever days	Detection criteria	Diagnostic assay	Disadvantage
1-7	Whole virus/bacteria	Cultures	➢ Cultures are subjected for contamination
Early, definitive diagnosis	Genetic materials (RNA/DNA)	Reverse Transcription Polymerase Chain Reaction /Polymerase Chain Reaction (RT-PCR/PCR)	➢ Needs sophisticated laboratories and expertise ➢ High cost ➢ Contamination
	Virus/bacteria encoded antigens	Antigen detection ELISA	➢ No reliable kit for CHIK and leptospirosis ➢ High cost for dengue kit
More than 7 days	Antibodies IgG	Haemaglutination Inhibition (HAI)-For CHIK and dengue Microscopic Agglutination Test (MAT)-For Leptospirosis	➢ Facilities are available at few reference laboratories ➢ Requires analyses of paired sera to achieve sufficient sensitivity ➢ Cumbersome and labor-intensive
	Antibodies IgM	Enzyme-Linked Immunosorbent Assay (ELISA) using whole cell viral (CHIK/dengue) or bacterial (leptospiral) antigen	➢ Health hazard though expose to infectious viruses/bacteria ➢ High production cost associated with virus bacteria cultivation ➢ Cross reactivity with other organisms of the same genus/family resulting false positivity

The need for developing cost effective, safe and simple diagnostic tests those combine with sensitivity and specificity have become ever more urgent to aid clinical case identification, management and to facilitate the implementation of rapid outbreak investigations. These infections can be laboratory diagnosed in the acute phase by detection of the virus/bacteria via cultures or a Polymerase Chain Reaction (PCR)-based assays, but most routine diagnosis relies on serologic methods. Cultures are subjected for contamination and required prolonged incubation period for all three diseases. PCR-based diagnostic methods need sophisticated laboratories and expertise. The reference standard serologic test, Haemagglutination Inhibition (HAI) assay for chikungunya and dengue and Microscopic Agglutination Test (MAT) for leptospirosis are inadequate for rapid case identification since they can only be performed in a few reference laboratories and require analyses of paired sera to achieve sufficient sensitivity. They are cumbersome and labor-intensive. Dependence upon the HAI/MAT results in delays in establishing the cause of outbreaks, as seen in

several investigations. Several Enzyme-Linked Immunosorbent Assay (ELISA) systems have been developed for serological tests after initial investigations of dengue/leptospirosis outbreaks by several researchers. Inhouse ELISAs require whole cell viral (dengue/chikungunya) or bacterial (leptospiral) antigen, which is often the limiting reagent in diagnostic assays and its large scale preparation is hindered by the cumbersome and expensive methods involving culture or in suckling mouse brain. Additionally, these methods result in crude extracts of variable quality with numerous non-specific antigens. Another problem is in a country where two or more flaviviruses/alphaviruse co-circulate, serodiagnosis is difficult due to the high serologic cross-reactivity. The recombinant protein-based antigens in ELISAs, produced by several researchers offer several distinct advantages over whole cell viral/bacterial antigens. First, the use of infectious virus for antigen production, which requires the highest level of microbiological security and a proper way to inactivate and to monitor the inactivation of the virus, is not required. Second, when antigens obtained from virus-infected cell extracts are used, it is difficult to standardize the test because of many factors, such as the multiplicity of virus infection, virus strains, cell line and cell condition, which may generate differences in the relative proportions of the immunoreactive proteins included in the antigen products. Hence the amounts of immuno reactive proteins may be different for different batches of antigen. This feature makes the quantitative interpretation of the test difficult. Therefore, the recombinant protein produced in bacteria/yeast system provides a solution to this problem, allowing easy standardization of antigen production. Third, the recombinant product can be obtained within a relatively short time (within 1 week after cloning), and the expression and purification procedures are simple and easy to perform. Fourth, low costs involved in growing bacteria/yeast producing recombinant protein. This procedure would be especially useful in cases of large-scale epidemiological investigations, as well as in developing countries where high-security laboratories are not available. The use of recombinant proteins for serodiagnosis of chikungunya and leptospirosis has not been widely investigated by researchers. Cloning and expression of a specific part of a viral/bacterial genes provides a straightforward alternative approach, simplifying purification and large-scale production of protein antigens for ELISAs. These recombinant protein antigen-based ELISA systems can be safe, affordable and specific tools for serodiagnosis of these three diseases in Sri Lanka.

ii. Scientific scope of the project

Overall objective

- To assist confirmation of disease outbreaks through developing competencies for ELISA-based rapid laboratory diagnosis for chikungunya, dengue and leptospirosis infections.

Specific objectives

- To prepare recombinant protein antigens for each disease.
- To develop ELISAs using recombinant protein antigens for each disease.
- To collect clinical samples from suspected patients for each disease.
- To analyze clinical samples by molecular and conventional diagnostic tests for each disease.
- To analyze serum samples confirmed by molecular and conventional assays by ELISAs developed using recombinant proteins.
- To document data on specificity and sensitivity of ELISAs developed by recombinant protein antigen with the gold standard/conventional assay.
- To calculate number of clinical samples analyzed for each disease by routing laboratory diagnostic service provided by ELISAs using recombinant protein antigens.
- To train project personnel in different diagnostic tests.
- To establish collaborative networks.
- To publish results of the project in peer-reviewed journals/presentations in local/ international conferences/meetings and thesis.

iii. Materials and methods

Work plan

The study was a 48 months' cross sectional prospective study with a 6-months pre-analytical phase, 36-months analytical phase and 6-months post analytical phase as described in the work plan (Annexure 3).

During pre-analytical phase of 6-months, collection of literature, obtaining ethical permission from the Ethical Review Committee of the Faculty of Medicine, University of Kelaniya, Sri Lanka, establishment of a collaborative network with reference laboratories, preparing data and sample collecting tools, obtaining pre-collected serum samples from the respective institutions, ordering procurement and establishment of molecular and conventional diagnostic assays for each disease were performed.

During the analytical phase of 36-months, preparation of reference materials used for standard serological assays, designing of novel recombinant protein antigens, construction of recombinant plasmids, cloning, expression and purification of recombinant proteins, assessment of reactivity of each protein towards antibodies using western blot analysis, development and optimization of in-house ELISAs using novel recombinant protein antigens using reference serum samples, establishment of standard general assays according to the World Health Organization (WHO) standards, preparation of panels of well characterized serum samples by testing samples collected from reference institutions using standard laboratory diagnostic assays and evaluation of in-house ELISAs developed using novel recombinant protein antigens on field collected serum samples were performed.

Analysis of data and writing the thesis were performed during the period of 6 months of post analytical phase.

This study was carried out in three different institutions following the work plan. Preparation of novel recombinant protein antigens and preliminary evaluations of reactivity of antigens were performed at the Recombinant Gene Products Group, International Centre for Genetic Engineering and Biotechnology, New Delhi Resident, India. Obtaining some of the panels of serum samples and preliminary testing of samples by standard serological methods and in-house ELISAs developed using novel recombinant protein antigens were performed at the Molecular Medicine Unit, Faculty of Medicine, University of Kelaniya, Sri Lanka. Characterization of panels of serum samples using standard assays recommended by the WHO and evaluation of recombinant protein antigens as diagnostic intermediates following WHO standards were performed at the Department of Virology, WHO Collaborative Centre for Viral Reference and Research, Institute of Tropical Medicine, University of Nagasaki, Japan.

Below activities were carried out in three counterpart institutions in 6 phases as mentioned in Table 2.

Table 2. Activities carries out in the study

Phase no	Activity	Place
Phase 1.	Preparation of recombinant protein antigens used as diagnostic intermediates	ICGEB, New Delhi
Phase 2.	Preparation well characterized serum panels for each disease	University of Kelaniya, Sri Lanka
Phase 3.	Preparation of serum panels to determine cut off values of ELISAs	University of Kelaniya, Sri Lanka
Phase 4.	Evaluation of recombinant protein antigens on clinical samples as diagnostic intermediates	Universities of Kelaniya, Sri Lanka and Nagasaki, Japan
Phase 5.	Carrying on common activities	University of Kelaniya, Sri Lanka
Phase 6.	Providing routine laboratory diagnostic facilities to the general public (University of Kelaniya, Sri Lanka)	University of Kelaniya, Sri Lanka
Phase 7.	Establishment of a collaborative net work	University of Kelaniya, Sri Lanka

Common methodologies followed for preparation of recombinant protein antigens for diagnosis of three diseases

Phase 1. Preparation of recombinant protein antigens used as diagnostic intermediates (RGP, ICGEB, New Delhi)

Recombinant protein antigens prepared for each disease by the RGP Group, ICGEB, New Delhi resident were transferred to the University of Kelaniya, Sri Lanka. They utilized institutional funds to prepare recombinant protein antigens for each disease. Few proteins for each disease were prepared separately and evaluated. Some of these proteins were prepared under two fellow ships given by the funds of the project and one direct fellow ships given by the ICGEB.

Design recombinant antigens: Few synthetic antigens of interest for each disease were selected based on literature. The genes were custom designed and chemically synthesized to prepare recombinant antigens. The choice of epitopes were based on three criteria namely, they were immunodominant, specific to IgM and/or IgG class antibodies and linear.

Construct recombinant plasmids: Plasmids were cleaved using relevant restriction enzymes and insert the gene of interest into the plasmid vector.

Clone, express and purify recombinant 6XHistidine (His) tagged proteins: Bacteria/yeast expression system were used to prepare His tagged proteins. Recombinant clones were selected on ampicillin plates and subjected to direct colony PCR screening, using insert specific primers, to identify recombinants harboring the synthetic gene. Orientation of the insert were verified by restriction analysis of plasmid minipreps.

Characterize each protein using western blot analysis: To characterize the purified recombinant protein, each protein was subjected to Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE, together with appropriate controls and prestained markers electroblotted onto nitrocellulose membrane and probed with penta-His monoclonal antibody and visualized using anti-mouse IgG-AP conjugate and BCIP/NBT substrate (AnandaRao *et al.*, 2005 and 2006).

Assess reactivity of each protein towards antibodies using western blot analysis: To assess the reactivity of recombinant protein towards anti-CHIK and dengue viruses and leptospirosis bacteria IgM and IgG antibodies, the purified protein was electrophoresed in a single wide well and blotted onto a nitrocellulose membrane, which was then cut into narrow strips. Each strip was then probed separately with

patient sera for each disease that was previously characterized to be IgM^+ IgG^+, IgM^+ IgG^-, IgM^- IgG^+ and IgM^- IgG^-. The secondary antibody-enzyme conjugate differed depending on the probing serum was used as the source of primary antibodies. For strips that was probed with IgM^+ IgG^- and IgM^- IgG^+ patient sera, the secondary antibody-enzyme conjugates used were anti-human IgM and anti-human IgG, respectively. For strips probed with IgM^+ IgG^+ and IgM^- IgG^- patient sera, either anti-human IgM- or IgG-enzyme conjugate were used (AnandaRao *et al.*, 2005 and 2006; Hapugoda *et al.*, 2007).

Develop and optimize inhouse ELISAs using recombinant proteins: To evaluate the possibility of using the recombinant protein for diagnosis of each disease, an indirect/direct IgG and IgM ELISA systems for human sera were developed and optimized using the recombinant protein as the antigen. In the optimization different assay conditions were used. Serum samples for each disease obtained from reference laboratories were used for development of inhouse ELISAs.

Train project personals: Research Assistant directly involved in the project was trained.

Phase 2. Preparation well characterized serum panels for each disease (University of Kelaniya, Sri Lanka)

Obtain (ethical) permission: This project proposal was submitted to the Ethical Review Committee of the Faculty of Medicine, University of Kelaniya, Sri Lanka. Permission for the collection of clinical samples from suspected patients warded at a relevant hospital/s was obtained from the Director and Consultant physician/s of the relevant hospital. Patients over 18 years were recruited after obtaining informed written consent. Venous blood from each patient was drawn using sterile precautions by a Medical Officer. Information obtained was strictly confidential and individual names were not be mentioned in the data entered in the computer and sample vials.

Establish a research team from the existing resources: An adequately sized of team with sufficient expertise was assigned to the project by the counterpart institution.

Prepare a proper transportation system: A proper transportation system for the collection of clinical samples from hospital/s and outbreak area/s was arranged.

Prepare data/sample collecting tools: Questionnaires, consent forms and a computer-aided traceability system for each patient from collecting till storage after testing were prepared. Equipment, consumables and others needed for collection of clinical samples were prepared.

Select sampling sites and human population: Depending on the epidemiological situation hospital/s from where a large number of suspected patients recorded were selected. Clinically suspected patients recorded from in/out patient departments of the relevant hospital/s were recruited according to the WHO criteria (WHO, 1997 and 2003).

Collect clinical, clinical laboratory information and exposure history from suspected patients: Clinical, clinical laboratory information and exposure history were collected from all suspected patients using an interviewer-administered questionnaire and by referring the bed head ticket for warded patients.

Collect clinical samples from suspected patients: An acute blood sample was collected from each patient in the early symptomatic phase when patients are at the hospital. A convalescent (follow-up) blood samples was collected after 7 and 14 days apart of collection of the acute blood sample by visiting to the hospital/patient's premise if they were discharged. Five millitres of venous blood sample was collected from each suspected patient into sterile disposable vials and transported directly to the main counterpart institution in ice. Serum was separated from each blood sample and stored frozen at -80^{o}C until tested.

Confirm suspected patients for each disease by laboratory diagnostic assays: In order to confirm suspected patients by laboratory diagnostic assays; molecular and antibody tests were performed on collected specimens as described in Table 3.

Table 3. Laboratory diagnostic assay performed

Type of sample	Type of assay	Disease and references
Acute (1-5 days fever)	CHIK-RT-PCR for CHIK	Hassebe *et al.* (2002)
	Dengue- RT-PCR for RNA	Chow *et al.* (1993)
	Nested PCR	Seah *et al.* (1995)
	Lepto-PCR for DNA	Gravekamp *et al.* (1993)
	Lepto-LAMP for DNA	Sonthayanon *at el.* (2011) Thermo usir Scientific kit
Convalescent (7-14 days apart of acute sample)	CHIK-IgM	Commercial kit (National Institute of Virology, Pune)
	CHIK-HAI	Clarke and Casals, 1958
	Dengue IgM and IgG	Commercial kit (Pan Bio, Australia)
	Dengue-HAI	Clarke and Casals, 1958
	Lepto-IgM	LEPTO CHECK IgM ELISA Commercial kit (Zephyr Biomedicals, India)
	Lepto-IgG	Leptospirosis: Martin and Pettit (1918)

Laboratory confirmed diseased and non-diseased patients among suspected patients were included in **Panels 1 and 2** respectively.

Phase 3. Preparation of serum panels to determine cut off values of ELISAs (University of Kelaniya, Sri Lanka)

Collect serum samples from healthy volunteers: Hundred and ten serum samples were collected from healthy volunteers living in non-endemic area/s for each disease. Further, they did not have a recent travel history to visit to an endemic area.

Confirm healthy volunteers: All serum samples were tested for antibodies by above mentioned laboratory diagnostic assays.

Determine the cut off Optical Density (OD) value: Serum samples were tested by ELISAs developed by novel recombinant protein antigens to determine the cut off OD value in each inhouse ELISA. Optimized assay conditions were used. One serum sample showing cut off OD value was selected

Phase 4. Evaluation of recombinant protein antigens on clinical samples as diagnostic intermediates (Universities of Kelaniya, Sri Lanka and Nagasaki, Japan)

Optimized assay conditions were used to evaluate IgM and IgG ELISAs using novel recombinant protein antigens. Well-characterized serum samples in Panels 1 and 2 were tested. A negative control, selected from Phase 3 was used to determine the cut off value.

Phase 5. Carrying on common activities (University of Kelaniya, Sri Lanka)

Enter and manage computer-aided data bases: Computer-aided data bases for record keeping and data analysis were established. Results of clinical, clinical laboratory, laboratory diagnostic assays and ELISAs performed using novel recombinant protein antigens were included into the data bases. All data were double entered in Excel spread sheets. Access to data was restricted to the investigators and research students.

Analyze research data: Differences in clinical and laboratory data obtained from disease suspected patients whenever possible were analyzed on the basis of the final diagnosis assigned on laboratory confirmation. Analysis was carried out by comparing clinical symptoms of all confirmed patients (Panel 1) with healthy people (Panel 2). Chi-squre test (Epi 6 Version 6.04d software, Centre for Disease Control, U.S.A.) was used for comparison of categorical data. Two variables were analyzed at a 95% confidence interval and p- value ≤ 0.05 was considered as significant.

In inhouse ELISA developed using recombinant proteins, cut off OD value for seropositivity was determined based on Mean OD + 3 standard deviations for negative control serum collected from healthy people in Phase 3. The mean and standard deviation of OD values were calculated using the SPSS 15.0 package. Serum samples which show OD value more than cut off value were considered as positive (Santos *et al.*, 2004).

Phase 6. Providing routine laboratory diagnostic facilities to the general public (University of Kelaniya, Sri Lanka)

Standard Operational Procedures (SOP) and safety manuals for diagnostic test developed for each disease using recombinant protein antigens were prepared and implemented. Resulted proteins are available at two counterpart institutions for field

use and further studies. Routine laboratory diagnostic services for CHIK, dengue and leptospirosis will be provided through IgM and IgG ELISAs developed using recombinant protein antigens.

Phase 7. Establishment of collaborative net works

National-1. Clinicians, Ministry of Health, Sri Lanka 2. Medical Research Institute, Sri Lanka.

International-1. Mammalian Biology-Recombinant Gene Products (RGP) Group, International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi resident 2. Dept. Of Virology (WHO Reference Centre for viral reference and research), Institute of Tropical Medicine, Nagasaki University, Nagasaki 3. Dept. of Biomedical Research, WHO/FAO/OIE and National Collaborating Centre for Reference and Research on Leptospirosis, Royal Tropical Institute, Amsterdam.

Methodologies followed for individual diseases (in detail)

For chikungunya

Phase 1. Preparation and purification of recombinant protein antigens

A. Selection and designing of genes

Expected properties of recombinant protein antigens used for IgM and IgG ELISAs

- Immunodominant (having exposed epitopes which can bind with target antibodies)
- Specific to detect CHIK IgM and IgG antibody
- Linear (no hidden epitopes, less folding ability, easy to purify)

CHIK E1 and E2 genes from CHIK genome (Figure 2) were selected to prepare recombinant protein antigens to detect anti–CHIK antibodies.

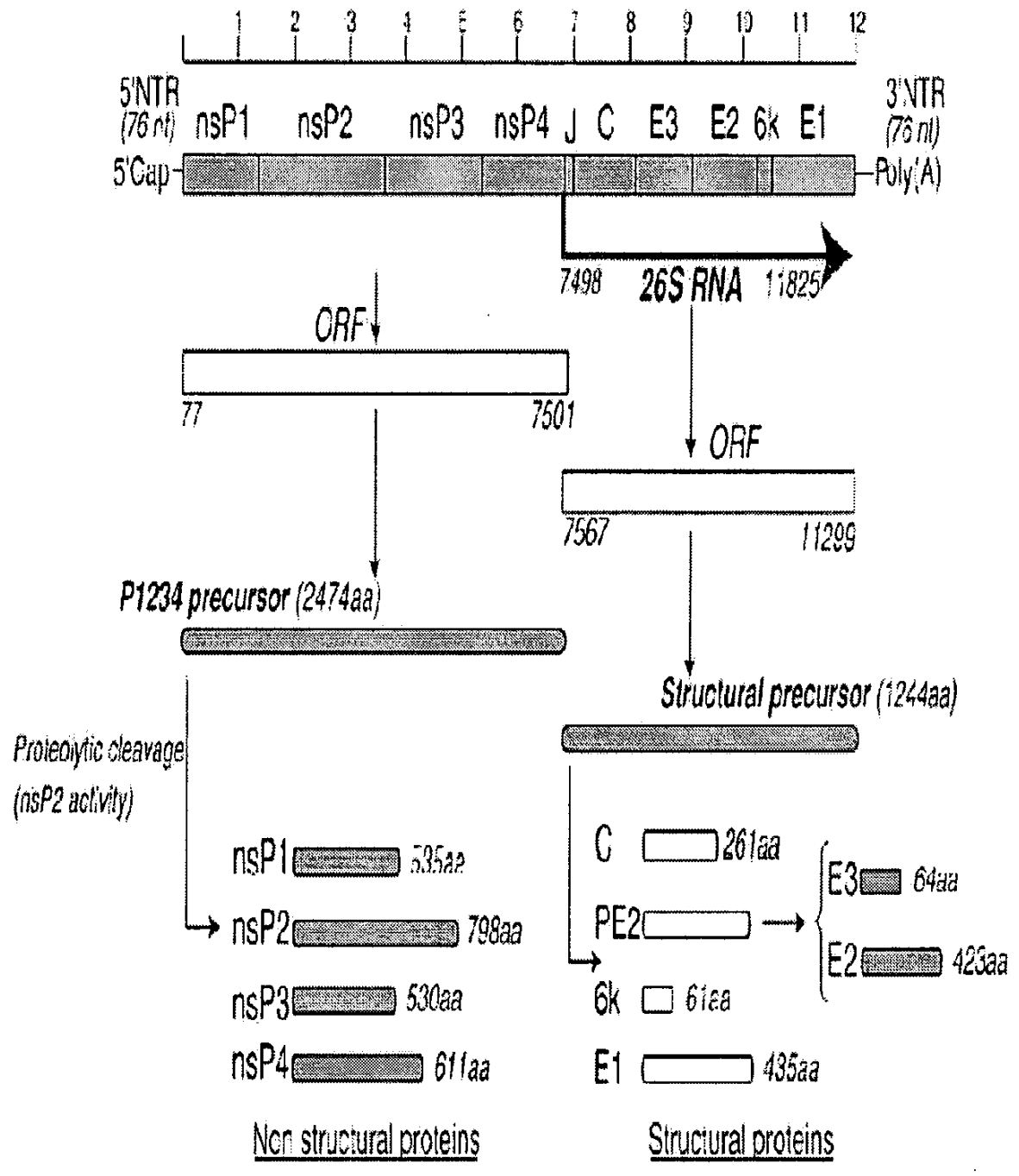


Fig. 2 Organization of the CHIKV genome and gene products. The CHIKV genome resembles eukaryotic mRNAs in that it possesses 5' cap structures and 3' poly(A) tail. The 5' and 3' proximal sequences of CHIKV genome carry non-translatable regions (NTR). The junction region (J) is also non-coding. A subgenomic positive-strand mRNA referred to as 26S RNA, is transcribed from a negative-stranded-RNA intermediate and serves as the mRNA for the synthesis of the viral structural proteins. The different non-structural proteins (nsP1-nsP4) and structural proteins (C, Capsid; E1, E2, E3, envelope; 6K) are generated after proteolytic cleavage of polyprotein precursors.

Outer layer of CHIK virus is covered with spikes (Figure 3). Triplets of heterodimer of E1 and E2 glyco proteins are components of a spike. The viral spike proteins (E1 and E2) facilitate attachment to cell surface and viral entry in to the cells (immunodominant). Properties of two proteins are given in Table 4.

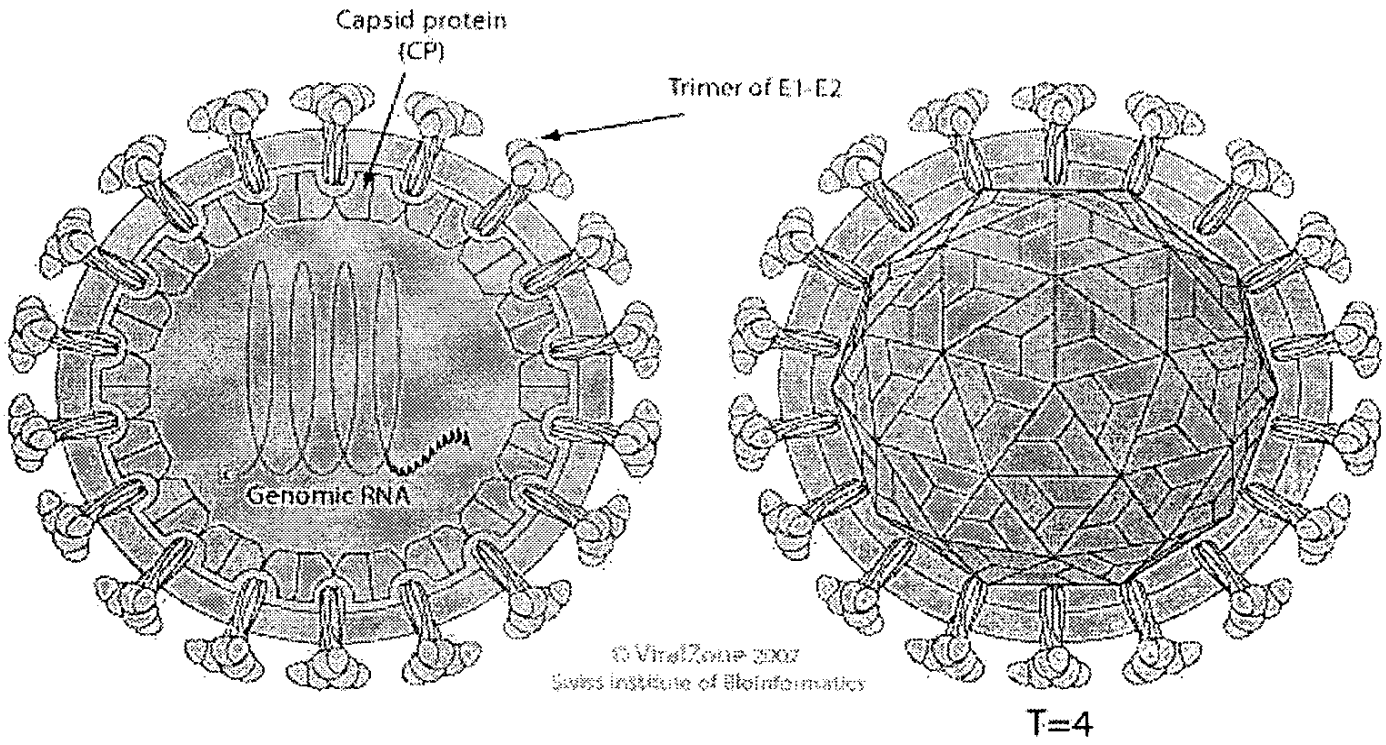


Figure 3. Outer layer and cross section of CHIK virus

Table 4. Properties of CHIK E1 and E2 proteins

Property	E1	E2
Size	435 amino acids MW of 44 kDa	423 amino acids MW of 43 KDa
Immunodominancy	Class II fusion protein that mediates low pH triggered membrane fusion during virus infection.	Type I trans membrane glycoprotein responsible for receptor binding during the course of *alphavirus* cycle
Epitope location	Fusion peptide is located at the tip of the E1 molecule in domain II, close to amino acid 226.	E2 interacts with the nucleo capsid core at the C terminus and contains the receptor binding site at the N terminus.

DNA sequences of these two genes were obtained from the Gene Bank (Figures 4A and B).

DNA sequence responsible to form 6X His tag at the N terminus of the protein was added to the E1 and E2 gene sequences. These two genes were chemically synthesized by DNA2.0 Inc.1430 Obrien Drive, Suite E, Menlo Park, CA, 94025.

Protein parameters were obtained using Gene runner, Expasy bioinformatics resource portal-Translate tool and Expasy bioinformatics resource portal-ProtParam Tool.

Start codon- Methionine

TCTGGCCATTCC**ATG**GGTCCTAATACTGTGGGTGTCCCGTACAAAACGCTGGTTAACCGTCCAGGTTA
CTCGCCGATGGTGCTGGAGATGGAGCTGCTGAGCGTCACCCTGGAACCGACGCTGTCCCTGGATTAC
ATCACGTGCGAGTACAAAACCGTTATTCCGAGCCCGTACGTGAAGTGCTGTGGCACCGCTGAATGCA
AGGACAAGAATTTGCCGGACTATAGCTGCAAAGTTTTCACCGGTGTCTATCCGTTTATGTGGGGCGG
TGCGTATTGCTTCTGCGATGCGGAAAACACCCAGTTGAGCGAGGCCCACGTTGAGAAAAGCGAGTCT
TGTAAAACGGAATTTGCCAGCGCGTACCGCGCACACACTGCATCCGCGAGCGCGAAGCTGCGTGTGC
TGTACCAGGGTAACAACATCACGGTGACGGCGTATGCAAACGGCGATCACGCTGTGACCGTCAAAG
ATGCGAAATTCATTGTCGGTCCGATGTCGAGCGCGTGGACCCCGTTCGATAACAAGATTGTTGTTTAC
AAGGGTGACGTGTACAACATGGACTATCCACCTTTTGGTGCAGGTCGTCCGGGCCAATTTGGCGACA
TTCAGAGCCGCACGCCGGAGAGCAAGGATGTTTATGCGAATACCCAGCTGGTCTTGCAGCGTCCGGC
AGCGGGTACGGTCCATGTTCCGTATAGCCAAGCACCGTCTGGTTTCAAGTATTGGCTGAAAGAACGT
GGTGCGAGCCTGCAACACACTGCTCCGTTTGGCTGTCAAATTGCGACCAATCCGGTGCGTGCGATGA
ATTGCGCGGTTGGCAATATGCCAATCAGCATTGATATTCCGGACGCAGCATTCACCCGTGTTGTCGAC
GCGCCAAGCCTGACCGACATGAGCTGCGAAGTCCCGGCGTGCACCCACTCCAGCGACTTCGGTGGTG
TCGCGATCATCAAGTACGCCGTGTCTAAGAAGGGTAAGTGTGCCGTGCACTCTATGACCAACGCTGT
TACGATTCGCGAGGCCGAAATCGAAGTCGAGGGTAACAGCCAGCTGCAAATCAGCTTTAGCACCGCT
CTGGCATCCGCTGAGTTTCGCGTTCAGGTTTGCAGCACCCAAGTGCACTGTGCGGCCGAGTGTCACCC
GCCTAAAGATCACATCGTGAACTACCCGGCGAGCCACACCACCCTGGGCGTGCAAGACATCAGCGC
AACGGCCATGTCCTGGGTTCAGAAAATCACCGGTGGTGGCGGTCATCATCACCACCACCATCACCAT
CATCATTGATAACTCGA

6X Histidine tag Stop codon

Figure 4A. Sequence of CHIK E1 gene (GENBANK-FJ513677.1)

Start codon - Methionine

CATGGGTTCGACCAAAGACAACTTCAATGTGTACAAAGCCACCCGCCCATATCTGGCGCACTGCCCG
GACTGCGGTGAAGGTCATTCGTGCCATAGCCCTGTGGCGCTGGAACGTATCCGTAACGAGGCGACCG
ATGGCACCCTGAAGATCCAAGTTAGCTTGCAAATCGGCATTGGTACGGACGACAGCCACGACTGGAC
CAAGCTGCGCTATATGGATAATCATATCCCGGCAGATGCCGGTCGTGCCGGTCTGTTTGTCCGCACCA
GCGCACCTTGCACCATTACGGGTACGATGGGTCACTTTATCCTGGCCCGTTGCCCGAAAGGCGAAAC
CTTGACCGTGGGCTTCACCGACTCTCGCAAAATCAGCCACAGCTGCACTCATCCGTTTCACCATGATC
CGCCGGTCATTGGTCGCGAGAAATTCCACAGCCGTCCGCAACACGGTAAAGAATTGCCTTGTAGCAC
GTATGTTCAGAGCAACGCGGCAACCGCTGAAGAGATTGAGGTTCACATGCCACCGGATACGCCGGA
CCGTACCCTGCTGTCTCAACAATCCGGCAATGTCAAGATTACGGTGAACAGCCAGACGGTCCGTTAC
AAATGTAATTGTGGCGGCAGCAACGAGGGCCTGATTACCACCGACAAAGTGATCAACAACTGTAAA
GTTGATCAGTGCCACGCAGCGGTGACCAATCACAAGAAGTGGCAGTACAATTCCCCGCTGGTGCCGC
GTAATGCGGAATTGGGCGATCGTAAAGGTAAGATTCACATCCCGTTCCCGCTGGCGAATGTTACTTG
TATGGTTCCTAAGGCTCGCAACCCAACGGTTACTTACGGTAAGAATCAGGTCATTATGCTGCTGTACC
CGGACCACCCGACCCTGCTGAGCTACCGCAGCATGGGTGAAGAACCGAATTACCAGGAAGAGTGGG
TTACGCACAAGAAAGAGGTTGTCCTGACGGTGCCGACCGAGGGTCTGGAGGTGACGTGGGGTAACA
ACGAACCGTACAAGTACTGGCCGCAACTGTCTGCGAACGGCACCGCGCATGGTCATCCGCACGAGAT
CATTCTGTATTACTATGAGCTGTATCCGACGATGACCGGTGGCGGTGGTCATCATCACCATCACCACC
ACCATCATCACTGATAACTCGA

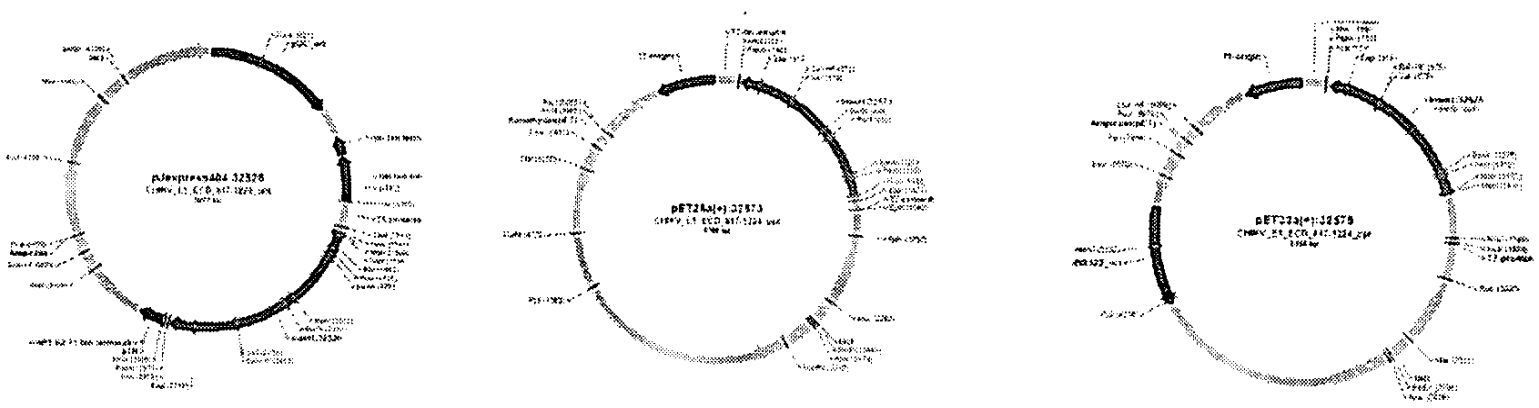
6X Histidine tag Stop codon

Figure 4B. Sequence of CHIK E2 gene (GENBANK-GU434112.1)

B. Clonning of genes to plasmids to form recombinant genes

These two gene sequences were cloned to expression vectors pET32a, pET28a and pJex 404 vectors. Six clones were prepared as described below (Figure 5). Some of the important properties of the vectors are given in Table 5.

E1/E2



pET 28a pJexpress 404

pET 32a

Figure 5. Plasmids used for cloning

Table 5. Properties of vectors

Vector	Resistence Marker	Promotor	Origin
pET 28a	Kannamycin	T7	pUC (High Copy)
pET 32a	Amphicilin	T7	pUC (High Copy)
pJexpress 404	Amphicilin	T5 *	pUC (High Copy)

C. Transformation of recombinant clones

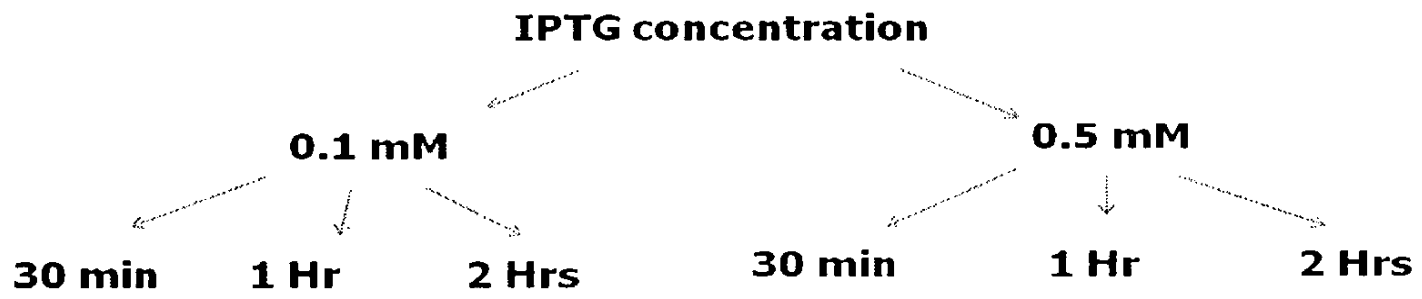
Six clones were transformed to a bacterial expression system, *Escherichia coli* (*E. coli*) (BL21) (DE3) separately using freeze thaw method.

D. Culturing of transformants

1ry culture- Six transformed bacteria cultures containing bacterial cells were cultured with appropriate antibiotic at 37 ^{0}C for overnight.

E. Expression of proteins

2ry culture was induced with 1 mM IPTG at 0.6 Optical Density (O.D) .Expression of protein was optimized varying the conditions (Figure 6) After induction cultures were kept at 16^{o}C for overnight. Cell pellets were washed with 0.9% NaCl. Induction was checked by running SDS PAGE gels followed by western blot analysis



Conditions: 16°C / 200 r.p.m

Figure 6. Optimization of expression was performed by induction with different IPTG conc. for different time intervals

F. Checking solubility of proteins

1 ml of pellet was dissolved in 1 ml of PBS with Lysozime. Sonicated for 2 minutes keeping the culture pellet at 4°C. Supernatant and pellet was run on a SDS PAGE Gel to check for protein (Figure 7).

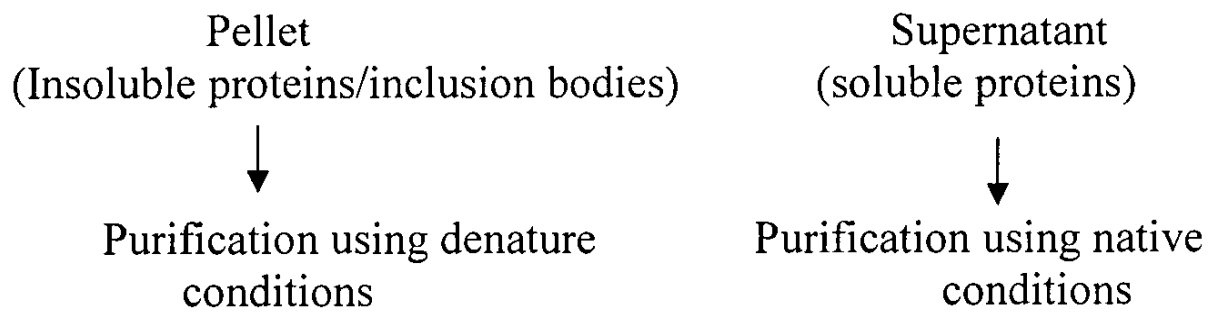


Figure 7. Schematic presentation of checking solubility of proteins

G. Purification of proteins

Protein purification is a three phase strategy (Figure 8). It involves binding of proteins to Ni-NTA columns, removing bulk impurities by washing with different concentration/pH buffers and finally eluting out the protein and refolding of proteins by dialysis. Protein purification was done using native conditions and denature conditions (Figure 9). It was done by Ni-NTA affinity chromatography (Figure 10) using fast Protein Liquid Chromatography (FPLC) (Figure 11).

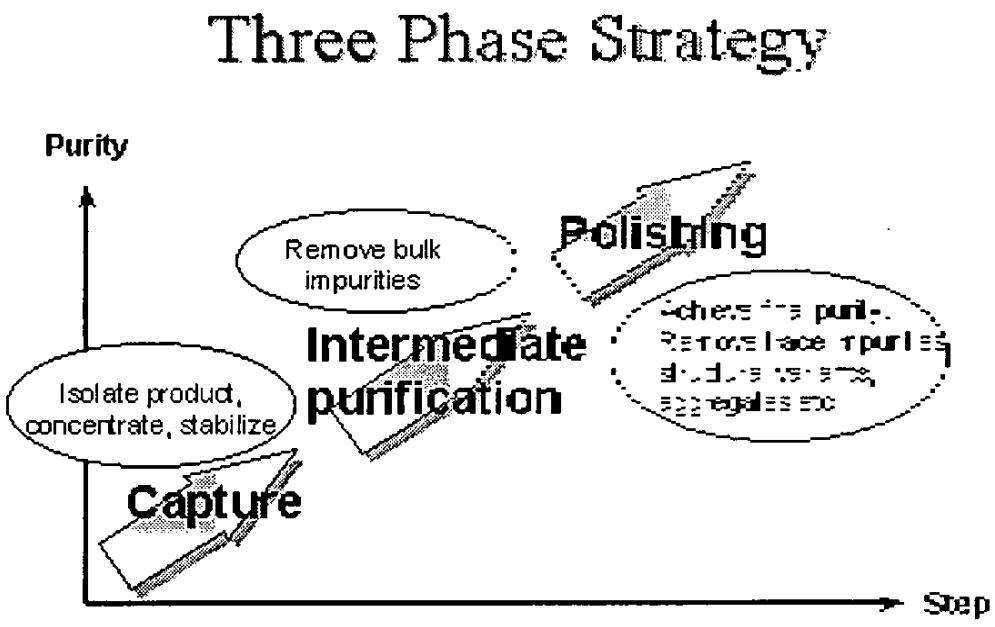


Figure 8. Steps of protein purification

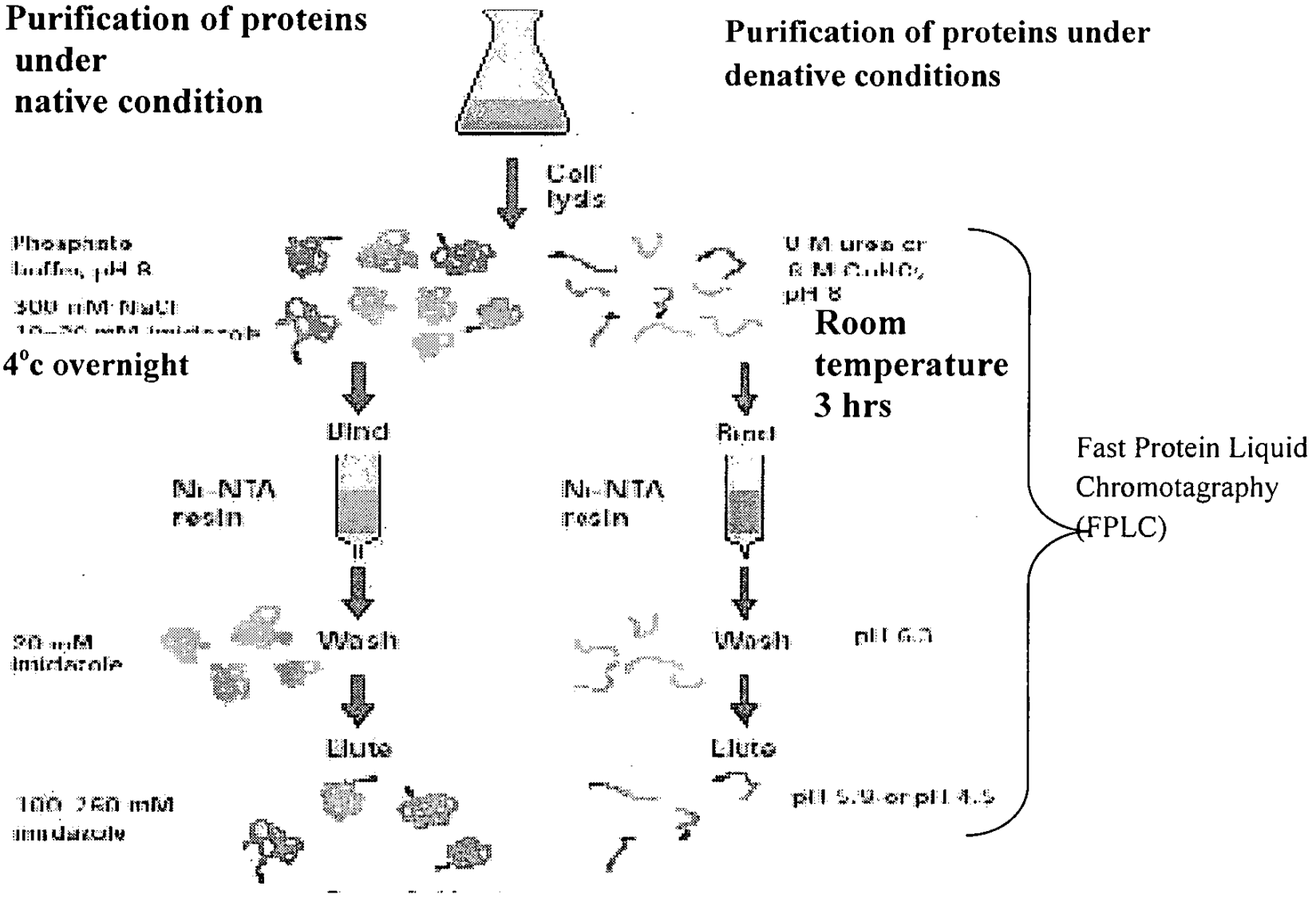


Figure 9. Methods used for protein purification

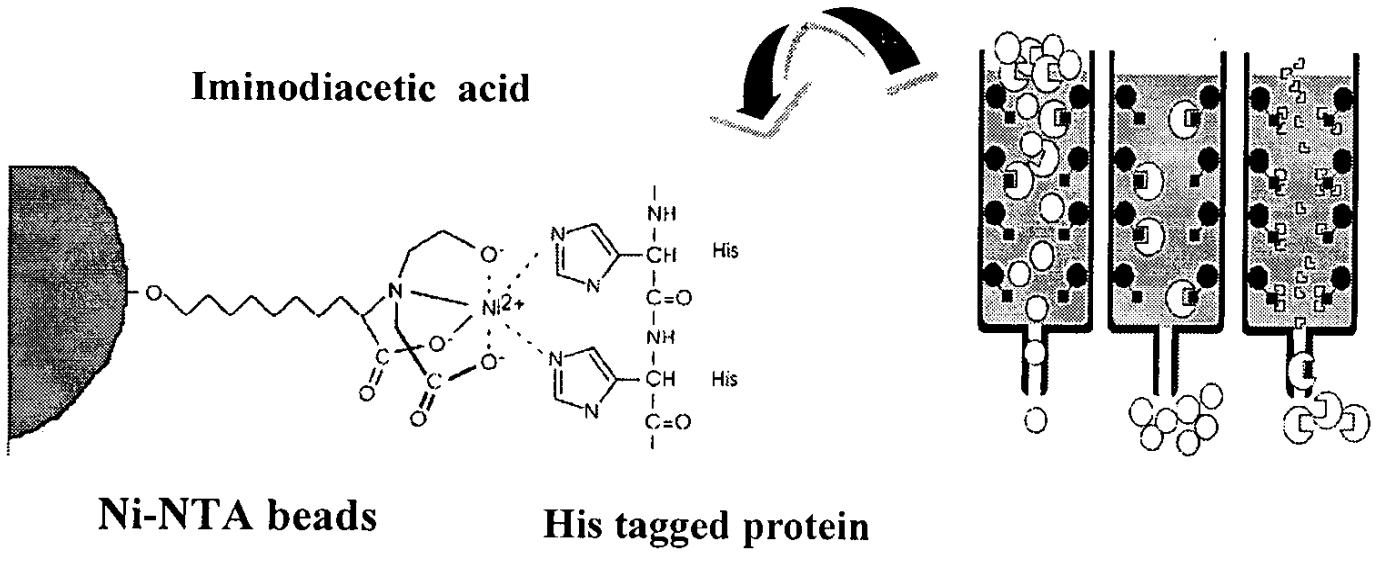
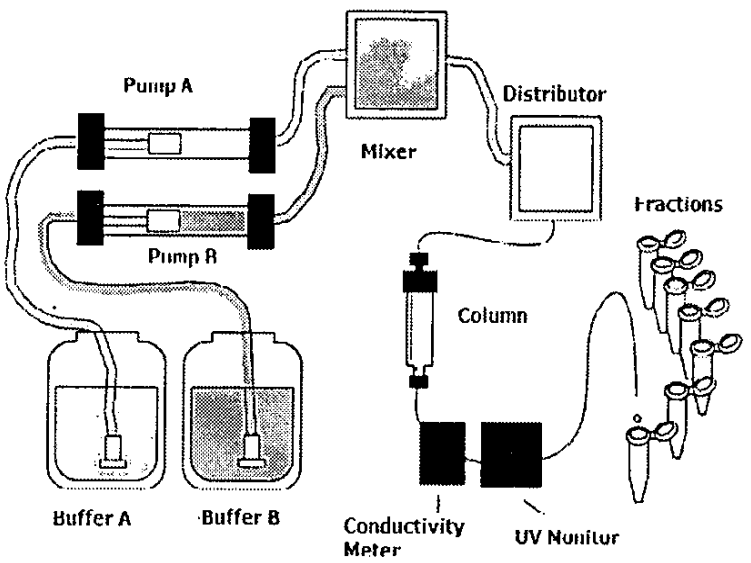


Figure 10. Theory of Ni- NTA affinity chromotagraphy



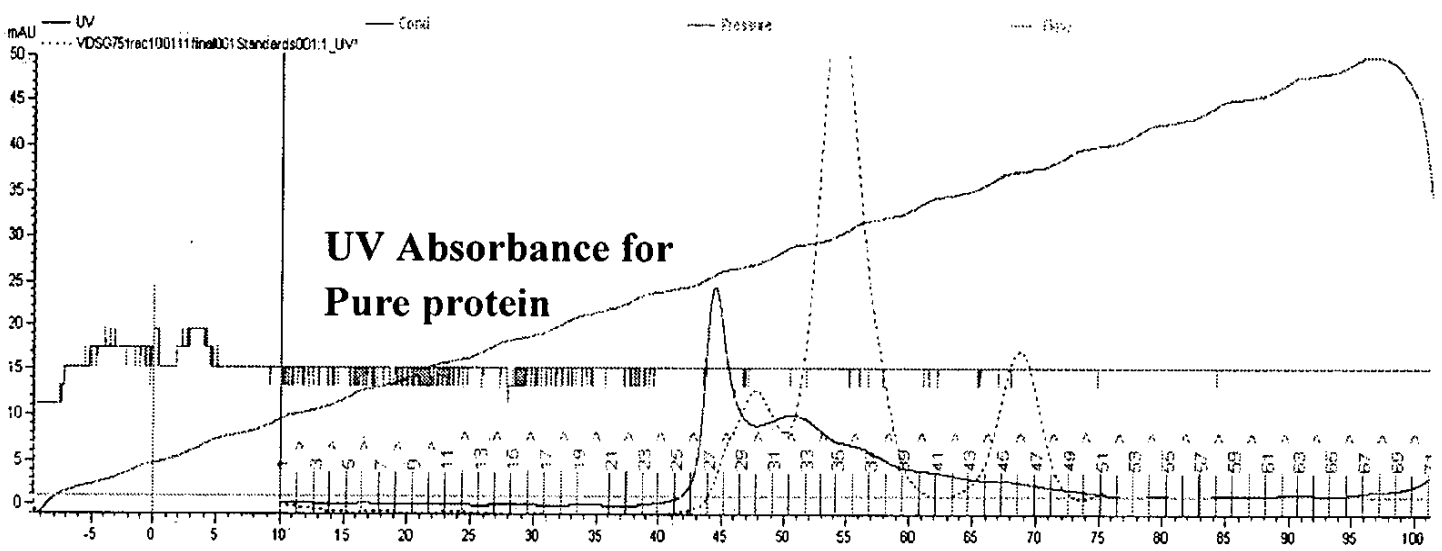


Figure 11. Function and output of Fast Protein Liquid Chromatography (FPLC)

Dialysis of E1 and E2 protein to remove buffers

Dissolved protein in 8 M urea was Introduced (2 ml) into a dialysis bag (Figure 12). It was dialyzed for 2 hrs at Room Temperature (RT) by stirring vs 6 M Urea, 50 mM Na_2CO_3, $NaHCO_3$ buffer. Then it was dialyzed for 2 hrs at Room Temperature (RT) by stirring vs 4 M Urea, 50 mM Na_2CO_3, $NaHCO_3$ buffer. Again the protein solution was dialyzed 2 hrs at 4°C by stirring vs 2 M Urea, 50mM Na_2CO_3, $NaHCO_3$ buffer. Finally, protein solution was dialyzed 2 hrs at 4°C by stirring vs 50 mM Na_2CO_3,

NaHCO3 buffer. After dialysis steps, protein mixture was centrifuged and supernatant was collected into a new tube. Absorbance of resulted refolded protein was measured at 280 nm.

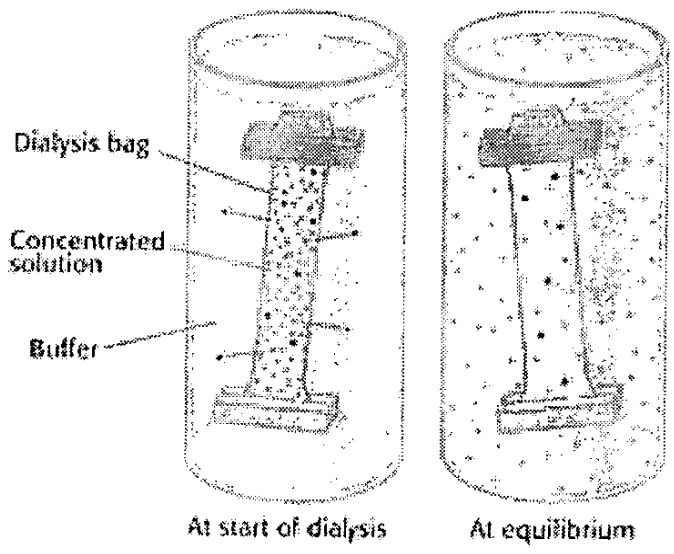


Figure 12. Dialysis of proteins

Phase 2. Development and optimization of IgM and IgG ELISAs using recombinant protein antigens to detect anti-CHIK antibodies

Samples- 15 positive and 5 negative serum samples confirmed by HAI (Medical Research Institute, Sri Lanka) and IgM ELISA (National Institute of Virology, Pune) Indirect IgM and IgG ELISAs (Figure 13) were optimized (Figure 14) using a checker board titration (Figure 15).

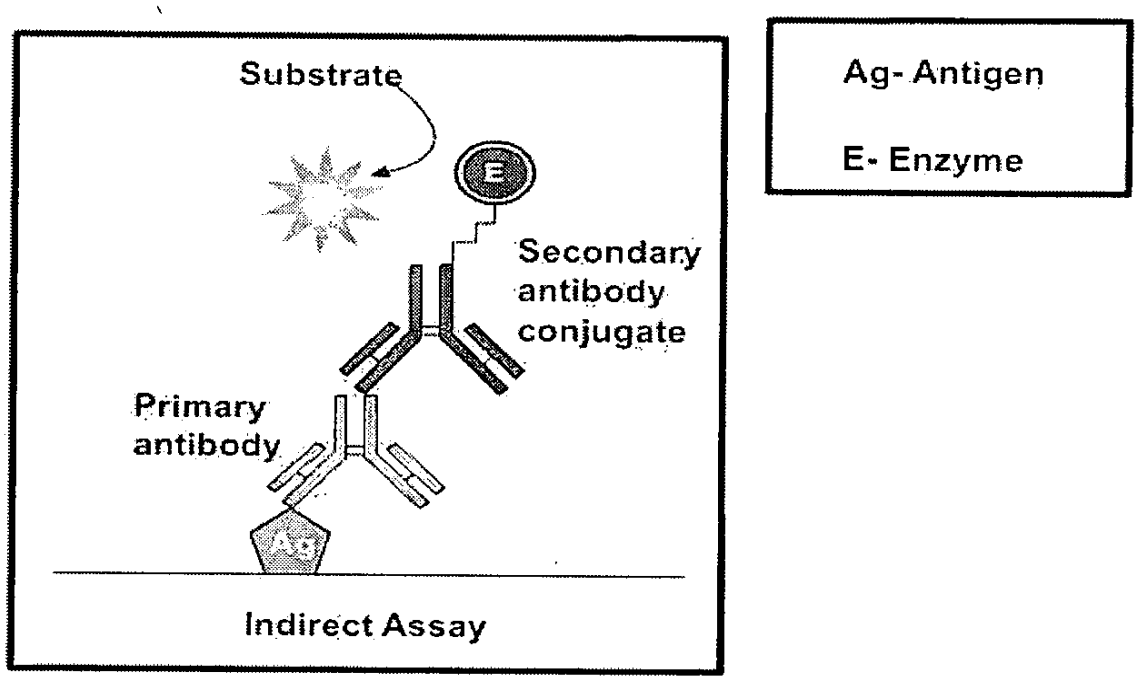
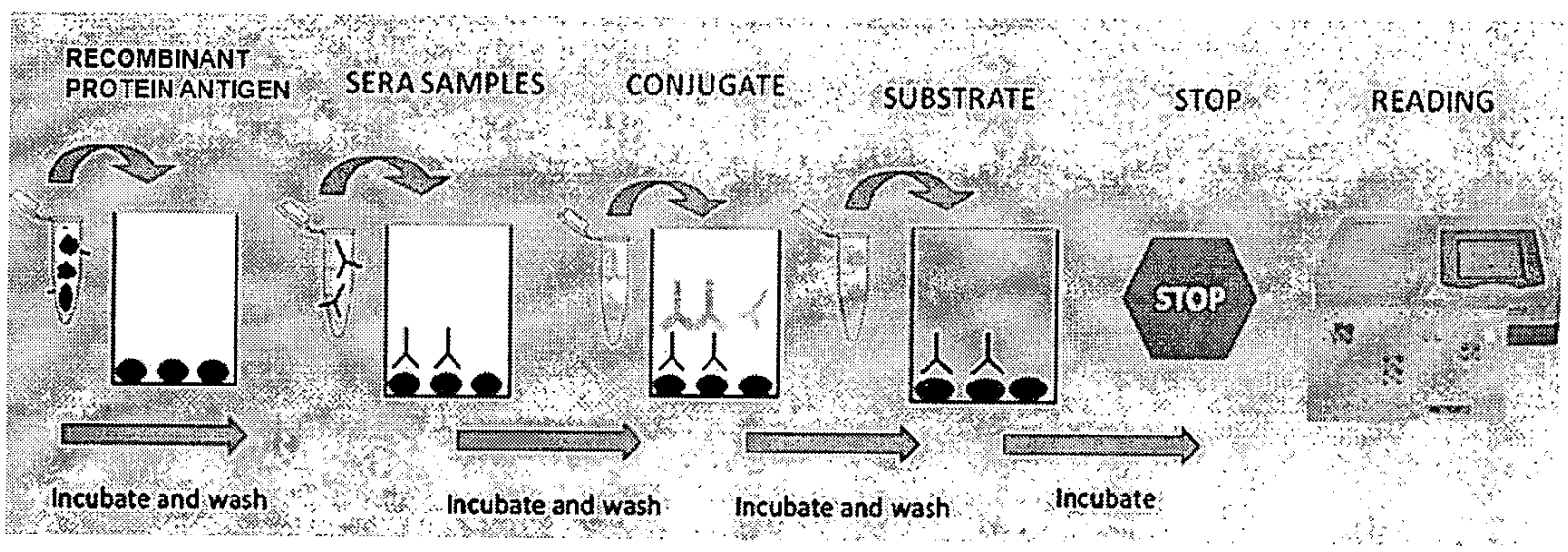


Figure 13. Theory of Indirect ELISA



Antigen coated with coating buffer	**1ry Antibody- Added serum samples Diluted in sera diluent (SD), 37°C 1 hr**	**2ry Antibody- Goat raised Anti Human Antibody (HRPO labeled) dissolved in SD, was added**	**3,3',5,5'- Tetramethyl benzidine substrate solution was added**	**2 N H_2SO_4 was a**	**Absorbance were taken at 650 nm ref 450 nm**

Figure 14. Basic optimization procedure of indirect ELISA

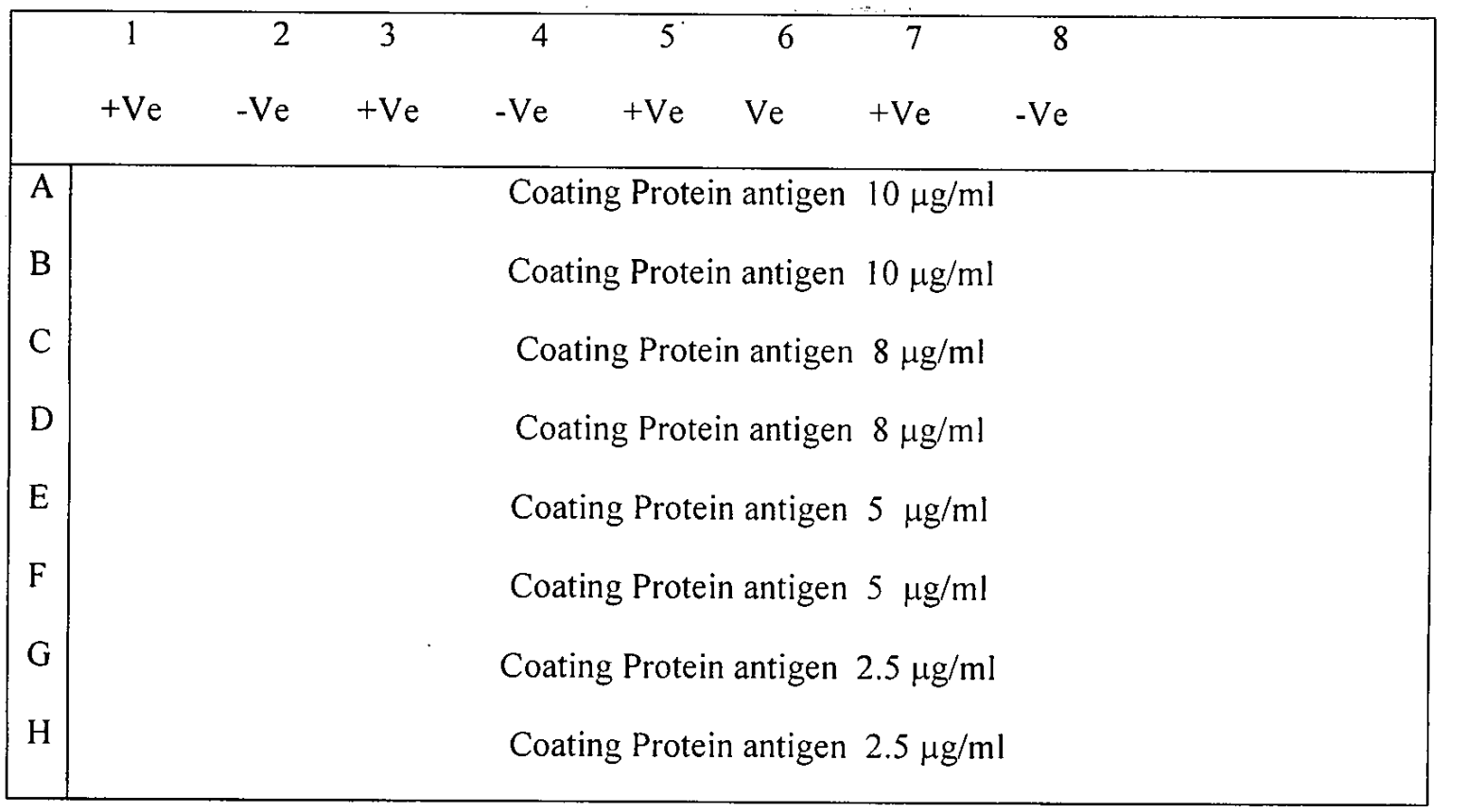


Figure 15. Checkerboard ELISA to determine optimal dilutions of protein antigens and secondary antibody for ELISA

Determination of cut off values for ELISAs 100 serum samples were Collected from healthy volunteers from Nuwara Eliya. Then confirmed samples for the absence of CHIK RNA (RT-PCR) and anti-CHIK antibodies (HAI). Tested samples by IgM and IgG ELISAs developed using recombinant protein antigens and determined cut off values for IgM and IgG ELISAs developed using recombinant protein antigens. Cut off value was determined as described in below equation.

Cut off value = Mean OD value of negative samples + 3 Standard Deviation (SD)

Phase 3. Evaluation of IgM and IgG ELISAs developed using recombinant protein antigens to detect anti-CHIK antibodies in field clinical samples

A panel of serum samples kindly provided by Medical Research Institute, Sri Lanka and Institute of Tropical Medicine, Nagasaki University were used. These samples were characterized using methods described in Table 2.

$$\text{Sensitivity} = \frac{\text{No of true positives}}{\text{No of true positives + false negatives}} \times 100$$

$$\text{Specificity} = \frac{\text{No of true negatives}}{\text{No of true negatives + false positives}} \times 100$$

Phase 4. Analysis of specificity, sensitivity and agreement of IgM and IgG ELISAs performed using each protein antigen with serological assays

Specificity, sensitivity and agreement of IgM and IgG ELISAs performed using each protein antigen with serological assays.

New experiments carried out (Not mentioned in the original proposal)
Cloning, expression and purification of E1 and E2 genes in *Pichia pastoris*

Both E1 and E2 genes, that code for the E proteins of CHIK virus, which should be cloned in *P. pastoris* was ordered from GenScript. These genes were cloned in to pPICZA vector (*Pichia* expression vector). Clones containing E1 and E2 insert were screened using Zeocin resistance. Plasmids were purified using these clones and was linearized using sacI enzyme. Linearized DNA was transformed to KM71H *Pichia* strain using electroporation. The transformed positive colonies were selected using zeocin resistance and was used in expression of E1 and E2 proteins. After confirming from a western blot these proteins were carried in to scale up expression. 1% methanol was used for 72 hours with every 12 hours induction. Cells were harvested at 72 hours. Cells were lysed using sonication through glass beads. Both the supernatant and pellet were checked by running a western blot. E1 and E2 proteins were purified under denature conditions (pH based) using Ni NTA columns.

IgM ELISA

Comparison of bacterial protein vs *Pichia* proteins: Serum samples for CHIK obtained from MRI was used for the development of inhouse ELISA. E1 and E2 bacterial proteins and *Pichia* proteins were coated in the same plate and previously optimized protocol was followed here for the comparison of proteins.

IgG ELISA

Comparison of bacterial protein vs *Pichia* proteins: Serum samples for CHIK obtained from MRI was used for the development of inhouse ELISA. E1 and

E2 bacterial and yeast proteins were coated in the same plate and previously optimized protocol was followed here for the comparison of proteins.

For leptospirosis

Phase 1. Preparation and purification of recombinant protein antigens
Leptospirosis Outer Membrane Lipoproteins (OMP) Lipl 32 (Figure 16) was selected as recombinant antigen genes. This gene was ordered and synthesized from Genscript, USA. Then, the gene was cloned and expressed in bacterial expression system *E. coli* (BL21) (DE3). Resulted protein was purified under denatured conditions using Ni- NTA.

ATCTCCGTTGCACTCTTTGCAAGCATTACCGCTTGTGGTGCTTTCGGTGGTCTGCCAAGCC
TAAAAAGCTCTTTTGTTCTGAGCGAGGACACAATCCCAGGGACAAACGAAACCGTAAAA
ACGTTACTTCCCTACGGATCTGTGATCAACTATTACGGATACGTAAAGCCAGGACAAGCG
CCGGACGGTTTAGTCGATGGAAACAAAAAAGCATACTATCTCTATGTTTGGATTCCTGCC
GTAATCGCTGAAATGGGAGTTCGTATGATTTCCCCAACAGGCGAAATCGGTGAGCCAGGC
GACGGAGACTTAGTAAGCGACGCTTTCAAAGCGGCTACCCCAGAAGAAAAATCAATGCC
ACATTGGTTTGATACTTGGATCCGTGTAGAAAGAATGTCGGCGATTATGCCTGACCAAAT
CGCCAAAGCTGCGAAAGCAAAACCAGTTCAAAA

Figure 16. Leptospira interrogans serovar Copenhageni strain, outermembrane lipoprotein (lipL32) gene sequence (GenBank: GQ204288.1)

Lipl48 protein was also prepared using above conditions.

Phase 2. Preparation well characterized serum panels for each disease (University of Kelaniya, Sri Lanka)

150 acute samples and 106 convalescent samples were collected and characterized using LAMP, PCR, IgM ELISA and MAT test as mentioned in Table 2.

Phase 3 (Preparation of serum panels to determine cut off values of ELISAs) and Phase 4 (Evaluation of recombinant protein antigens on clinical samples as diagnostic intermediates) were performed following the same procedures as mentioned in the original methodology in the project.

For dengue Dengue

Phase 1. Preparation well characterized serum panels for each disease (University of Kelaniya, Sri Lanka)

206 acute samples and 202 convalescent samples were collected and characterized using tests as mentioned in Table 2.

Phase 2. Field evaluation of recombinant protein antigen and kits

ELISA kits (IgM-Lot no. EDM 020810, IgG-EDG010710) prepared by J. Mitra Company, transferred through ICGEB, New Delhi were field evaluated. Serum samples and results were transferred to ICGEB, New Delhi Resident for their studies.

iv. Results/out puts

For Chikungunya

Preparation of CHIK proteins in bacterial vector systems- *E. coli*

Protein parameters

Protein parameters of E1 and E2 recombinant proteins in pET28a, pET32a and pJex404 vectors are given in Figures 17-22.

ExPASy — ProtParam

ProtParam

User-provided sequence:

```
        10         20         30         40         50         60
MCDNITVCVDY RELVNMDCYS DHVLEMEELS VILEPTLSLD YITCEYKTVI DSPYVKCCCT

        70         80         90        100        110        120
AECKDNNLPD YSCKVFTGVY PFMWGGAYCF CDAENTQLSE AHVEKSESCK TEFASAYRAH

       130        140        150        160        170        180
TASASAKLRV LYQGNNITVT AYANGDHAVT VKDAKFIVGP MSSAWTPFDN KIVVYKGDVY

       190        200        210        220        230        240
NMDYPQFGAG SPGQFKMSTQ HTPHSHVLVK NTGHVTVQPH MKLGGAKLTS

       250        260        270        280        290        300
ERGASLQHIA REGTQIAINP VRAWNCAVGN MPISIDIPDA AFIRVVDAPS LTDMSCEVPA

       310        320        330        340        350        360
CTHSSDFGGV AILKYAVSKK GKCAVHSMTN AVTIREAEIE VEGNSQLQIS FSTALASAEF

       370        380        390        400        410        420
RVQVCSTQVH CAAECHPPKD HIVNYPAAHT TLGVQDISAT AMSWVQKITG GVGLIVAVAA

HHH
```

Number of amino acids: 423

Molecular weight: 45931.0

Theoretical pI: 6.59

Figure 17. protein parameters of E1 protein in pET28a

ExPASy ProtParam

ProtParam

User-provided sequence:

```
        10         20         30         40         50         60
MSDKIIHLTD DSFDTDVLKA DGAILVDFWA EWCGPCKMIA PILDEIADEY QGKLTVAKLN

        70         80         90        100        110        120
IDQNPGTAPK YGIRGIPTLL LFKNGEVAAT KVGALSKGQL KEFLDANLAG SGSGHSMGPN

       130        140        150        160        170        180
TVGVPYKTLV NRPGYSPMVL EMELLSVTLE PTLSLDYITC EYKTVIPSPY VKCCGTAECK

       190        200        210        220        230        240
DKNLPDYSCK VFTGVYPFMW GGAYCFCDAE NTQLSEAHVE KSESCKTEFA SAYRAHTASA

       250        260        270        280        290        300
SAKLRVLYQG NNITVTAYAN GDHAVTVKDA KFIVGPMSSA WTPFDNKIVV YKGDVYNMDY

       310        320        330        340        350        360
PPFGAGRPGQ FGDIQSRTPE SKDVYANTQL VLQRPAAGTV HVPYSQAPSG FKYWLKERGA

       370        380        390        400        410        420
SLQHTAPFGC QIATNPVRAM NCAVGNMPIS IDIPDAAFTR VVDAPSLTDM SCEVPACTHS

       430        440        450        460        470        480
SDFGGVAIIK YAVSKKGKCA VHSMTNAVTI REAEIEVEGN SQLQISFSTA LASAEFRVQV

       490        500        510        520        530
CSTQVHCAAE CHPPKDHIVN YPASHTTLGV QDISATAMSW VQKITGGVGH HHHHHHHHH
```

Number of amino acids: 539

Molecular weight: 58289.1

Theoretical pI: 6.21

Ext. coefficient 64290

Abs 0.1% (=1 g/l) 1.103, assuming all Cys residues are reduced

Figure 18. protein parameters of E1 protein in pET32a

ProtParam

User-provided sequence:

```
        10         20         30         40         50         60
EVKRMKGHSM GPNTVGVPYK ILVNKPGYSP MVLEMELLSV TLKPTLSLDY IKCEYKTVIP

        70         80         90        100        110        120
SPYVKCCGTA ECKDKNLPDY SCKVFTGVYP FMWGGAYCFC DAENTQLSEA HVEKSESCKT

       130        140        150        160        170        180
EFASAYRAHT ASASAKLRVL YQGNNITVTA YANGDHAVTV KDAKFIVGPM SSANTPFDNK

       190        200        210        220        230        240
IVVYKGDVYN MDYPPFGAGR PGQFGDIQSR TPESKDVYAN TQLVLQRPAA GTVHVPYSQA

       250        260        270        280        290        300
PSGFKYWLKE RGASLQHTAP FGCQIATNPV RAVNCAVGNM PISIDIPDAA FTRVVDAPSL

       310        320        330        340        350        360
TDMSCEVPAC THSSDFGGVA IIKYAVSKKG KCAVHSMTNA VTIREAEIEV EGNSQLQISF

       370        380        390        400        410        420
STALASAEFR VQVCSTQVHC AAECHPPKDH IVNYPASHTT LGVQDISATA MSWVQKITGG

       430
GGHHHHHHHH HH
```

Number of amino acids: 432

Molecular weight: 46894.1

Theoretical pI: 6.66

Ext. coefficient 50310
Abs 0.1% (=1 g/l) 1.073, assuming all Cys residues are reduced

Figure 19. protein parameters of E1 protein in pJEX

ProtParam

User-provided sequence:

```
        10         20         30         40         50         60
MGSIKINFNV YKATRPYLAH CPDCGEGHSC HSPVALERIR NEATDGTLEI QVSLQIGIGT

        70         80         90        100        110        120
DDSHDWTKLR YMDNHIPADA GRAGLFVRTS APCTITGDMG HPILARCPKG ETLIVGFIDS

       130        140        150        160        170        180
RKISHSCTHP FHHDPPVIGR EKFHSRPQHG KELPCSTYVQ SNAATAEEIE VHMPPDIPDR

       190        200        210        220        230        240
TLLSQQSGNV KIIVNSQIVR YKCNCGGSNE GLIITDKVIN NCKVDQCHLA VTNHKKWQYN

       250        260        270        280        290        300
SPLVPRNAEL GDRKGKIHIP FPLANVTCMV PKARNPTVTY GKNQVIMLLY PDHPTLLSYR

       310        320        330        340        350        360
SMGEEPNYQE EWVTHKKEVV LTVPTEGLEV TWGNNEPYKY WPQLSANGTA HGHPHEIILY

       370        380
YYELYPTMTV GGGHHHHHHH HHH
```

Number of amino acids: 386

Molecular weight: 42898.3

Theoretical pI: 7.42

Ext. coefficient 51340
Abs 0.1% (=1 g/l) 1.197, assuming all Cys residues are reduce

Figure 20. protein parameters of E2 protein in pET28a

ProtParam

ProtParam

User-provided sequence:

```
        10         20         30         40         50         60
MSDKIIHLTD DSFDTDVLKA DGAILVDFWA EWCGPCKMIA PILDEIADEY QGKLTVAKLN

        70         80         90        100        110        120
IDQNPGTAPK YGIRGIPTLL LFKNGEVAAT KVGALSKGQL KEFLDANLAG SGSGHSMGPN

       130        140        150        160        170        180
TVGVPYRTLV NRFGYSEMVL EMELLSVTLE PTLSLDYITC EYKTVIPSPY VKCCGTAECK

       190        200        210        220        230        240
DKNLPDYSCK VFTGVYPFMW GGAYCFCDAE NTQLSEAHVE KSESCKTEFA SAYRAHTASA

       250        260        270        280        290        300
SAKLRVLYQG NNITVTAYAN GDHAVTVKDA KFIVGPMSSA WTPFDNKIVV YKGDVYNMDY

       310        320        330        340        350        360
PPFGAGRPGQ FGDIQSRTPE SKDVYANTQL VLQRPAAGTV HVPYSQAPSG FKYWLKERGA

       370        380        390        400        410        420
SLQHTAPFGC QIATNPVRAM NCAVGNMPIS IDIPDAAFTR VVDAPSLTDM SCEVPACTHS

       430        440        450        460        470        480
SDFGGVATIK YAVSKKGKCA VHSMTNAVTI REAEIEVEGN SQLQISFSTA LASAEFRVQV

       490        500        510        520        530
CSTQVHCAAE CHPPKDHIVN YPASHTTLGV QDISATAMSW VQKITGGGGH HHHHHHHHH
```

Number of amino acids: 539

Molecular weight: 58289.1

Theoretical pI: 6.21

```
Ext. coefficient    64290
Abs 0.1% (=1 g/l)   1.103, assuming all Cys residues are reduced
```

Figure 21. protein parameters of E2 protein in pET32a

ProtParam

User-provided sequence:

```
        10         20         30         40         50         60
EVKHMGGHSM GSIKDNFMVY KATPFYLARC PDCGSGHSCH SFVALERIRN EATDGILKIQ

        70         80         90        100        110        120
VSLQIGIGTD DSHDWTKLNY MDNHIPADAG KAGLFVRTSA PCTIIGTMGH FILARCPKGE

       130        140        150        160        170        180
TLTVGFTDSR KISHSCTHPF HHDPPVIGRE KFHSRPQHGK ELPCSTYVQS NAATAEEIEV

       190        200        210        220        230        240
HMPPDTPDRT LLSQQSGNVK ITVNSQTVRY KCNCGGSNEG LITTDKVINN CKVDQCHAAV

       250        260        270        280        290        300
TNHKKWQYNS PLVPRNAELG DRKGKIHIPF PLANVTCMVP KARNPTVTYG KNQVIMLLYP

       310        320        330        340        350        360
DHPTLLSYRS MGEEPNYQEE WVTHKKEVVL TVPTEGLEVT WGNNEPYKYW PQLSANGTAH

       370        380        390
GHPHEIILYY YELYPTMTVG GGHHHHHHHH HH
```

Number of amino acids: 392

Molecular weight: 43861.3

Theoretical pI: 7.53

Ext. coefficient	51340
Abs 0.1% (=1 g/l)	1.171, assuming all Cys residues are reduced

Figure 22. protein parameters of E2 protein in pJex 404

Expression of recombinant proteins

Expression of proteins were checked by using SDS-PAGE and western blot.

Expression of E1 and E2 proteins of each clone are shown in SDS-PAGE gels given below (Figures 23-25). Expression of E1 and E2 proteins in each clone are shown in western blot (Figures 26).

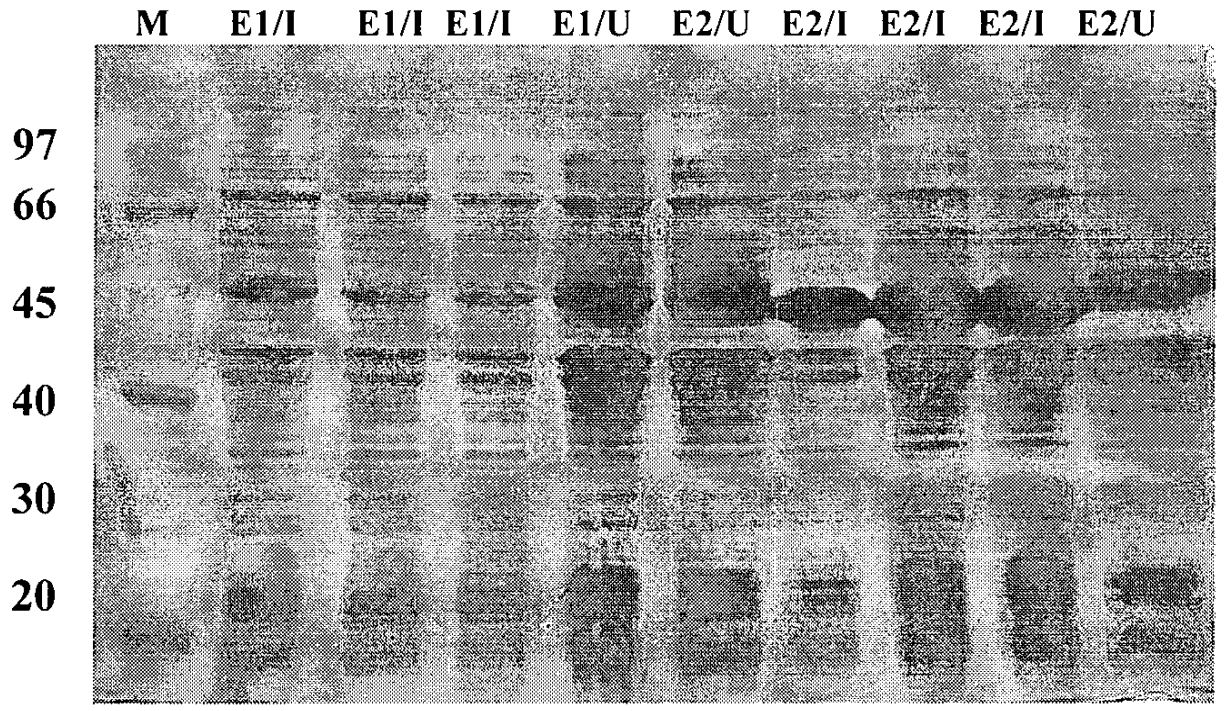


Figure 23. SDS PAGE gel photo showing induction with 1 mM IPTG , pET 28a E1 and E2.

M- Low Molecular Weight marker, E1/I - Induced E1 protein, E1/U - Uninduced E1 protein, E2/I - Induced E2 protein, E2/U - Uninduced E2 protein

E1 was not expressed → Checked with western blot

M.W 45.93 Kda
pI 6.59

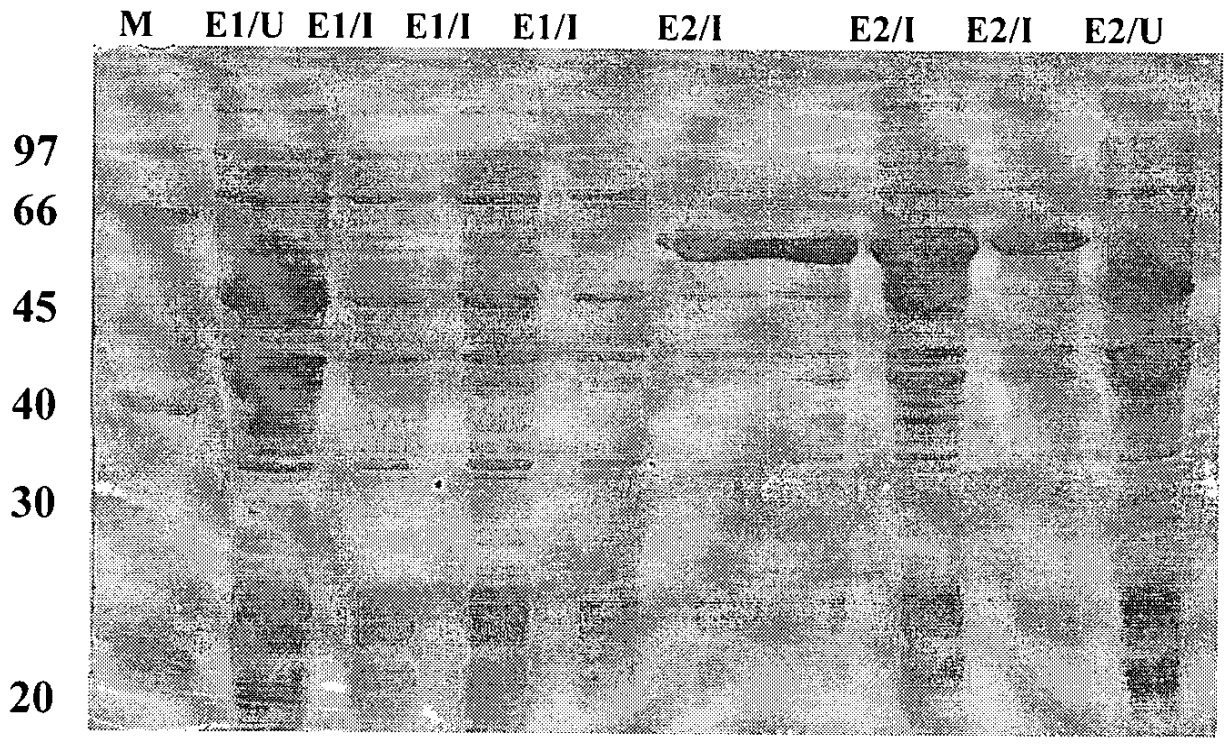


Figure 24. SDS PAGE gel photo showing induction with 1 mM IPTG- pET 32 E1 and E2

M- Low Molecular Weight marker, E1/I - Induced E1 protein, E1/U - Uninduced E1 protein, E2/I - Induced E2 protein, E2/U - Uninduced E2 protein

E1 was not expressed → Check with western blot
M.W 58.289
pI 6.21

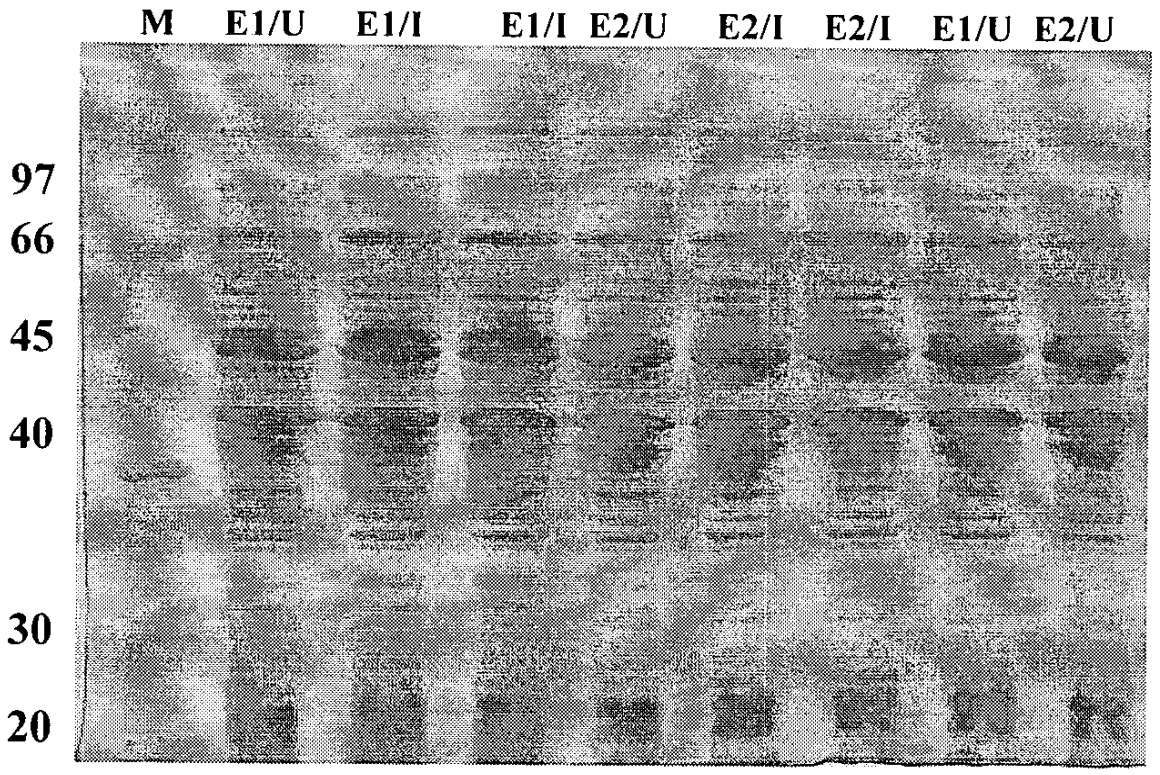


Figure 25. SDS PAGE gel photo showing induction with 1 mM IPTG- pJex E1 and E2

M- Low Molecular Weight marker, E1/I - Induced E1 protein, E1/U - Uninduced E1 protein, E2/I - Induced E1 protein, E2/U - Uninduced E1 protein

E1 and E2 genes were not expressed ➡ Check with western blot

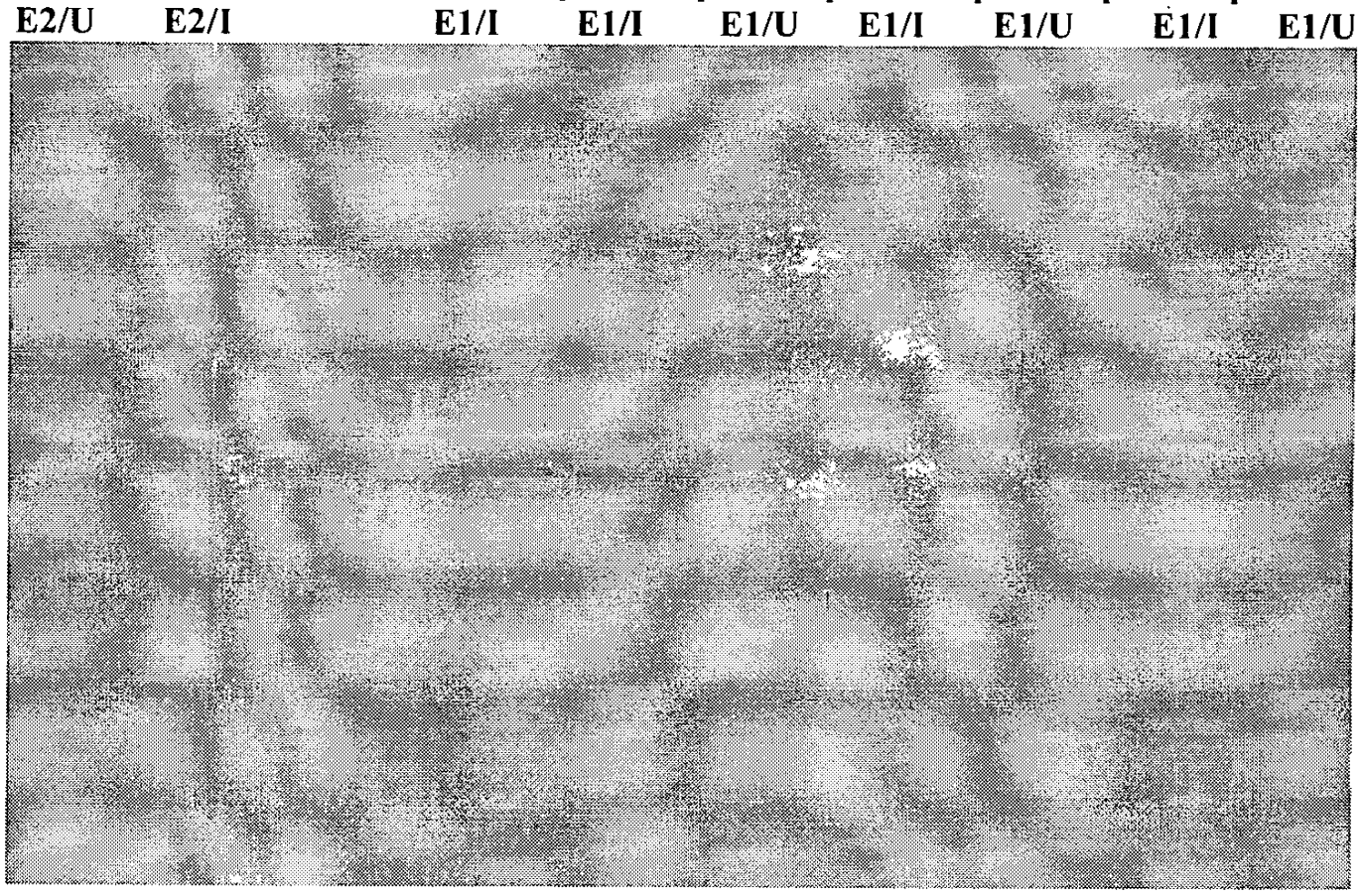


Figure 26. Western blot image showing expression of E1 and E2 proteins

PSM- Pre stained marker, E1/I - Induced E1 protein, E1/U - Uninduced E1 protein, E2/I - Induced E2 protein, E2/U - Uninduced E2 protein

Checking solubility of E1 and E2 proteins

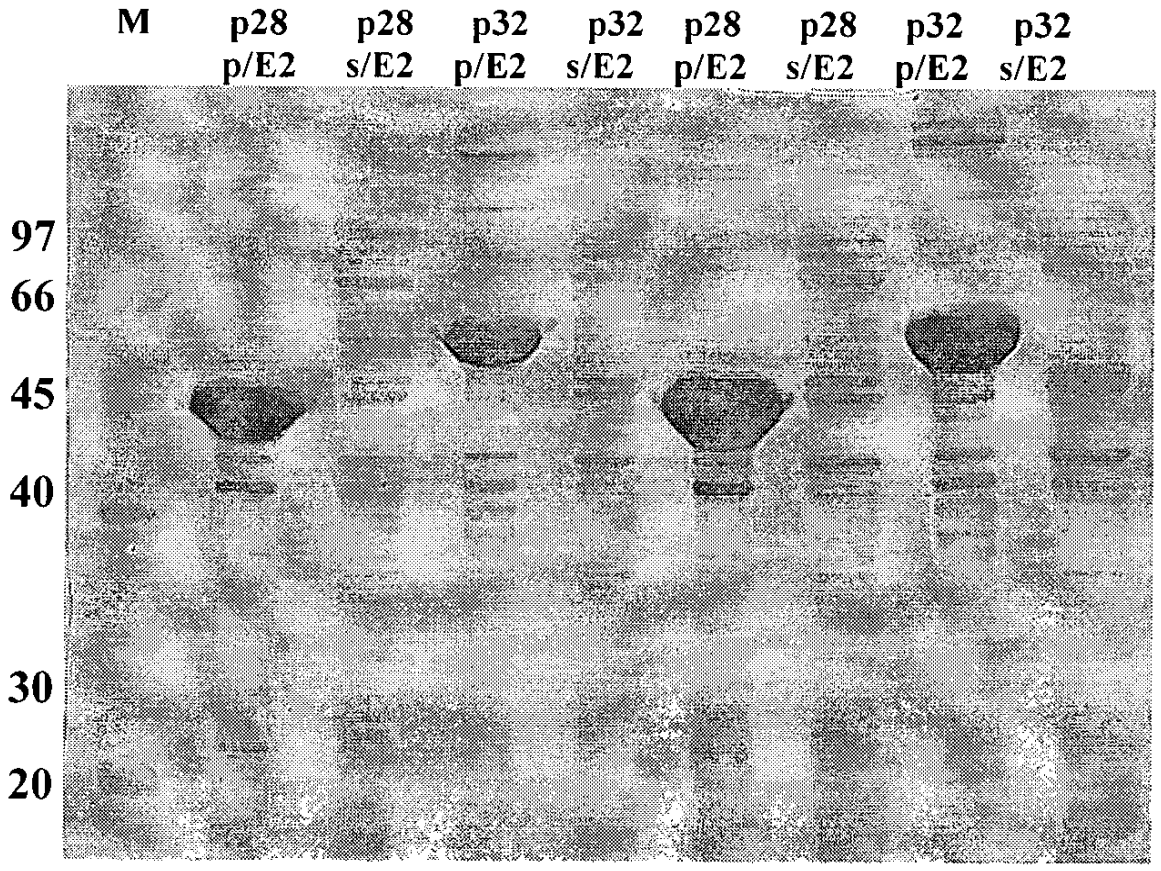


Figure 27. SDS PAGE gel photo showing solubility of E2 protein

M- Low Molecular Weight marker, P28 – pET28, P32 – pET32, p/E2 – E2 protein in pellet, s/E2 – E2 protein in

Induction of proteins at different IPTG concentration and time periods

Induction of E2 and E1 proteins are shown in Figures 28 and 29 respectively and results are tabulated in Table 5.

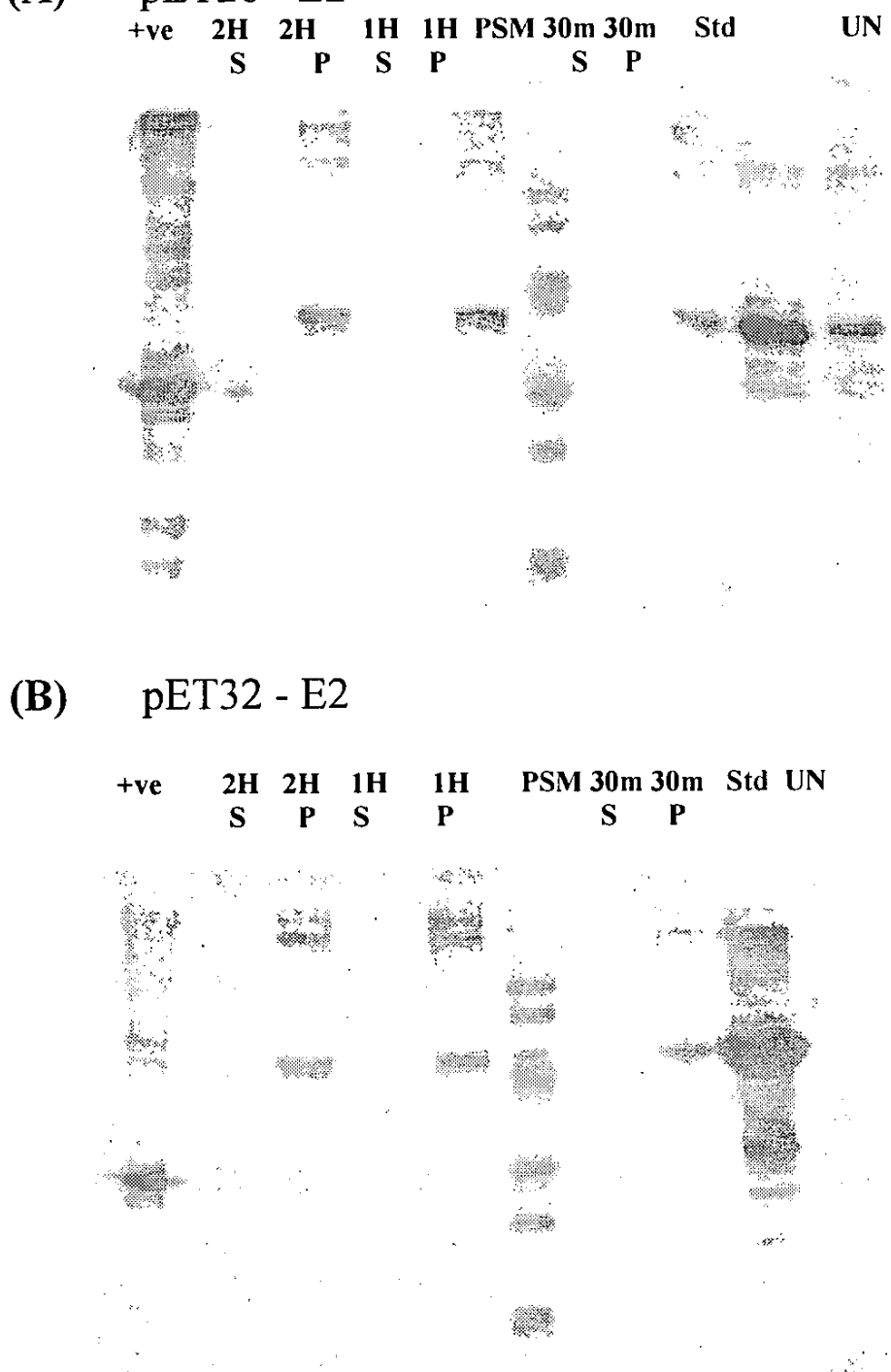
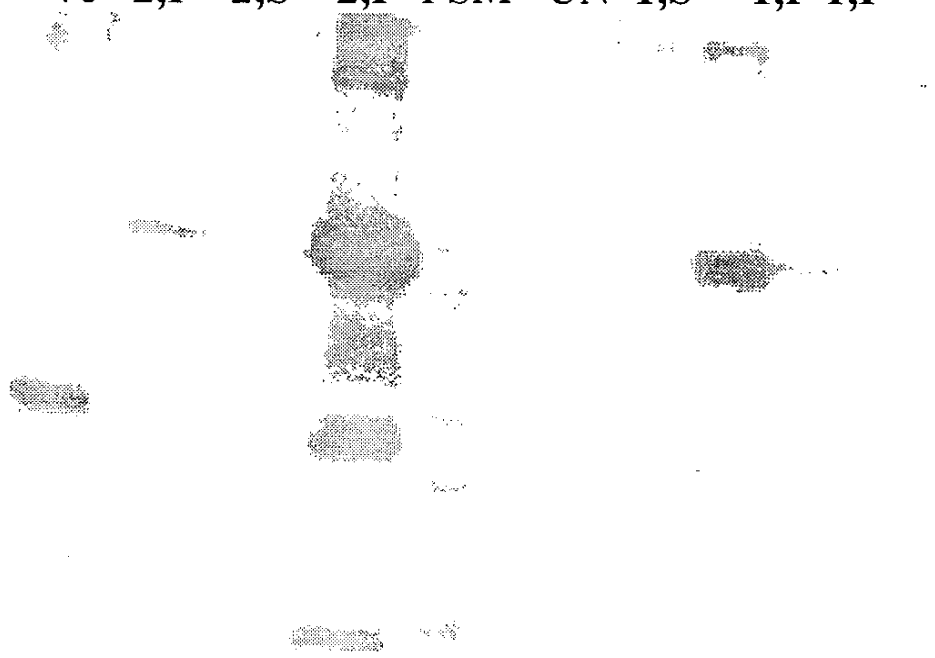


Figure 28.Western Blot images showing expression of E2 protein at different IPTG concentrations, (A) pET28a- E2 ,(B) pET32a-E2

+ve - positive marker, 2H/S- Protein amount in supernatant after IPTG induction for 2hrs, 2H/P- Protein amount in pellet after IPTG induction for 2hrs, 1H/S Protein amount in supernatant after IPTG induction for 1hr, 1H/P- Protein amount in pellet after IPTG induction for 1hr, 30m/S- Protein amount in pellet after IPTG induction for 30 mins, 30m/S- Protein amount in pellet after IPTG induction for 30 mins, Std- standard method 1mm IPTG for 3 hrs. Time change affect the solubility or yield of the proteins. Standard method is more suitable.

(A) pET32- E1



(B) pET28- E1

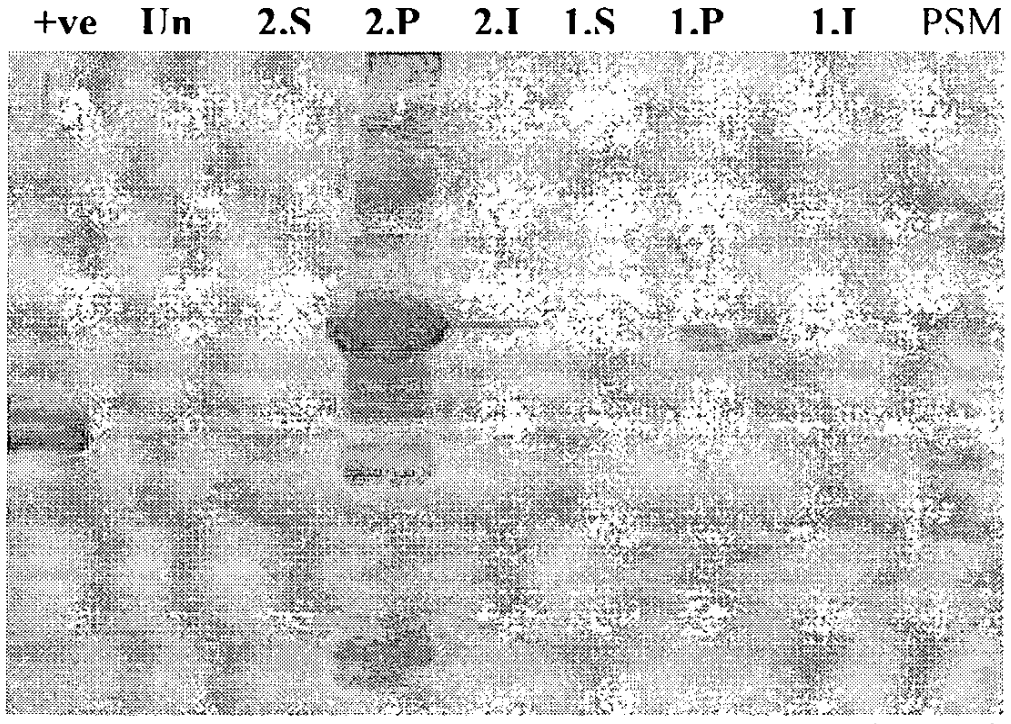


Figure 29.Western Blot image showing expression of E1 protein at different IPTG concentrations, (A) pET32a-E1,(B) pET28a- E1

PSM- pre stained marker+ve- positive marker, 1- 1st method. 2- 2nd method, S- supernantant, P- pellet, UN- uninduced, I- Induced, 1st method- 1μM IPTG 16C, 24HRs 2nd method - 1mM IPTG, 37C, O.D 0.6 – 16C, 24Hrs Yield for protein is more in 2nd method Therefore the standard method is more suitable

Table 6. IPTG concentration Vs. induction of proteins

IPTG concentration	Conditions	Visibility of protein E1	Visibility of protein E2
1 mM	37°C, 3 Hrs	Good	Very Good
0.5 mM	37°C, 30 min	-	Good
	37°C, 1 Hr	-	Good
	37°C, 2 Hr	-	Good
1 uM		-	Poor

Optimal assay conditions for purification of proteins under native condition

During protein purification different concentration of buffers, temperatures, time duration, sonication were optimised for getting better yield of each protein. Optimal assay conditions under native (Figures 30 and 31) and denative (Figures 32 and 33) conditions are given below.

- Resuspending cell pellet in Lysis buffer (50 mM Sodium phosphate, 30 mM NaCl, 20 mM Imidazole, 0.2% tween 20, Lysosyme 1 mg/ml, pH 8.0)
- Chilling at -20°C for 15 min
- Stirring at 4°C for 10 mins
- Sonicating at 33% amplitude for 5 mins
- Pelleting cell debris at 10,000 rpm for 1 hour
- Equilibrating supernatant with Ni- NTA at 4°C over night
- Purification using FPLC with Imidazole elution

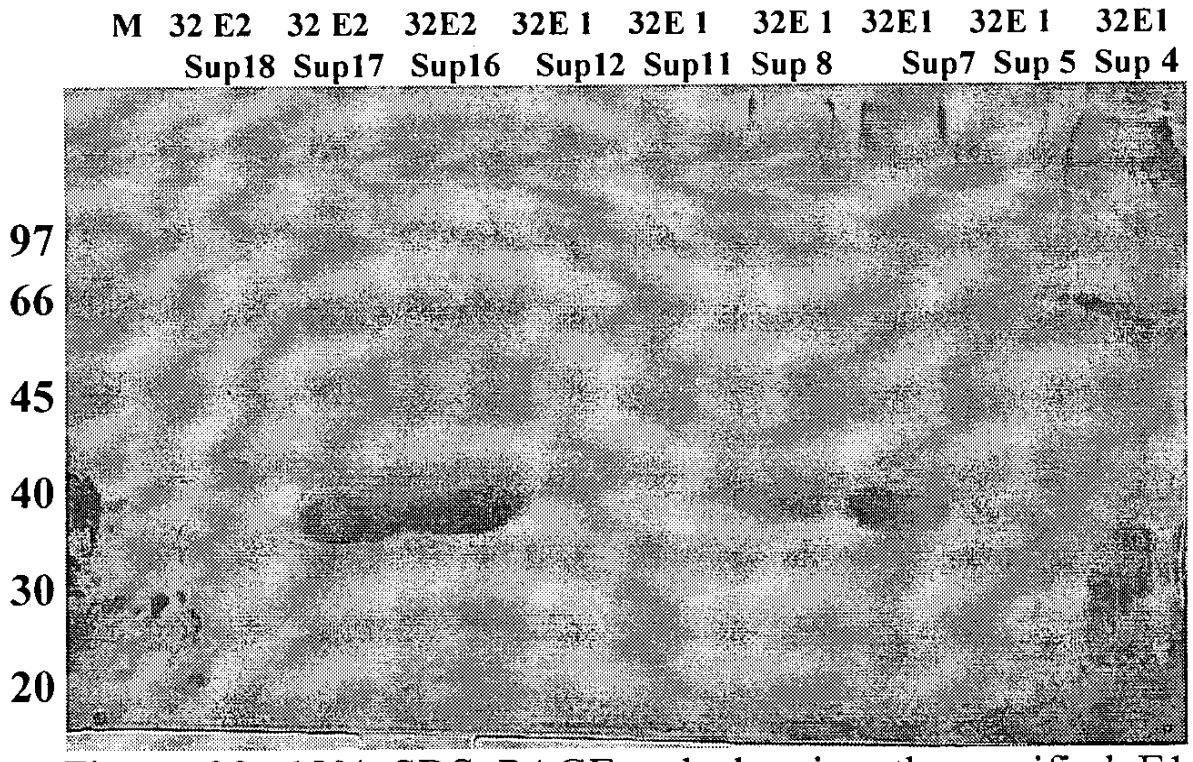


Figure 30. 12% SDS PAGE gel showing the purified E1 and E2 protein: Under native condition

M –Low Molecular Weight Marker, Sup 4 – sup18 protein fractions

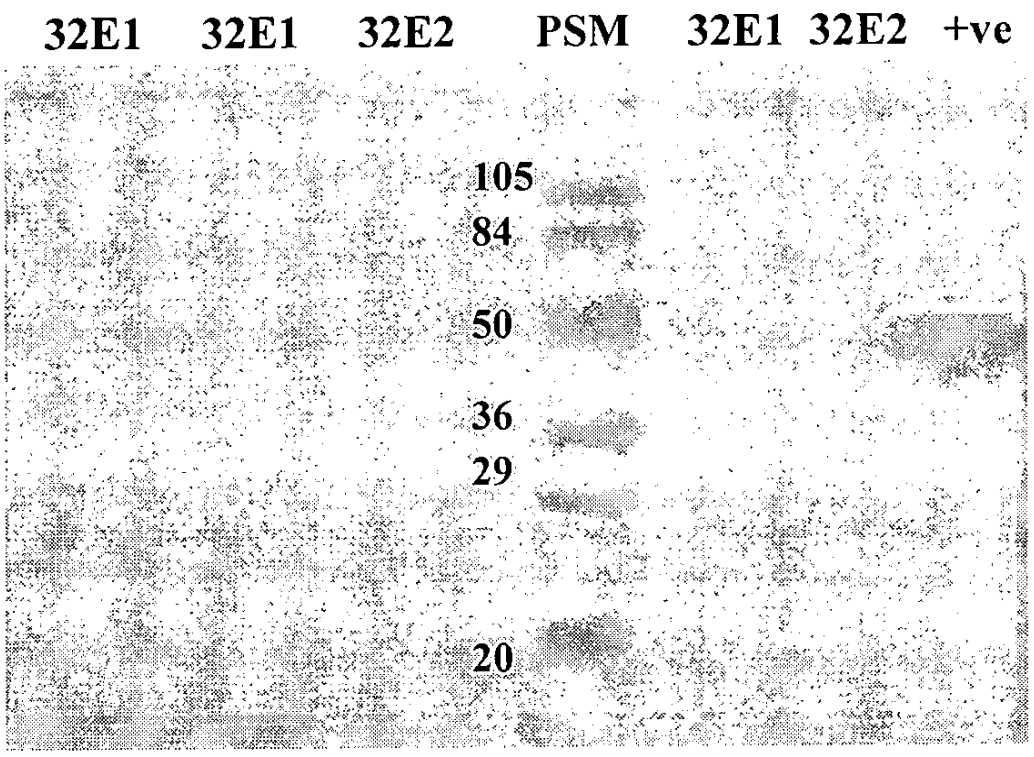


Figure 31. Western blot showing the purified E1 and E2 protein-Under native condition

PSM –Pre Stained Marker, +ve - positive control.

Purification under denature condition
Optimal conditions for purification proteins under denative conditions

- Resuspending cell pellet in Lysis buffer (10mm Tris, 300 mM NaCl, 6M GuHCL, 0.5% tween 20, pH 8.0)
- Stirring at RT until getting a homogeneous solution
- Sonicating at 33% amplitude for 5 mins
- Pelleting cell debris at 10,000 rpm for 1 hour
- Equilibrating supernatant with Ni- NTA at RT for 4 hours.
- Purification using FPLC with pH based elution method.
- Checking protein using a SDS- PAGE gel

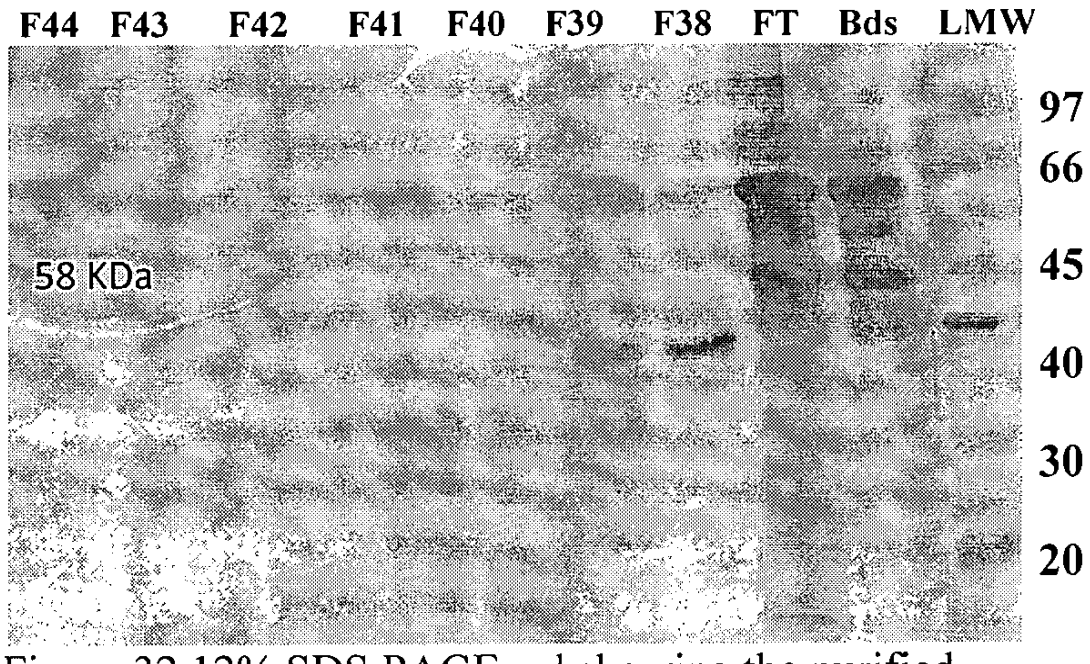


Figure 32.12% SDS PAGE gel showing the purified E2 protein: under denative conditions LMW – Low Molecular Weight Marker, Bds- Beads, FT- Flow Through, F38 – F41 protein fractions.

Concentration of E1 protein 0.21 mg/ml

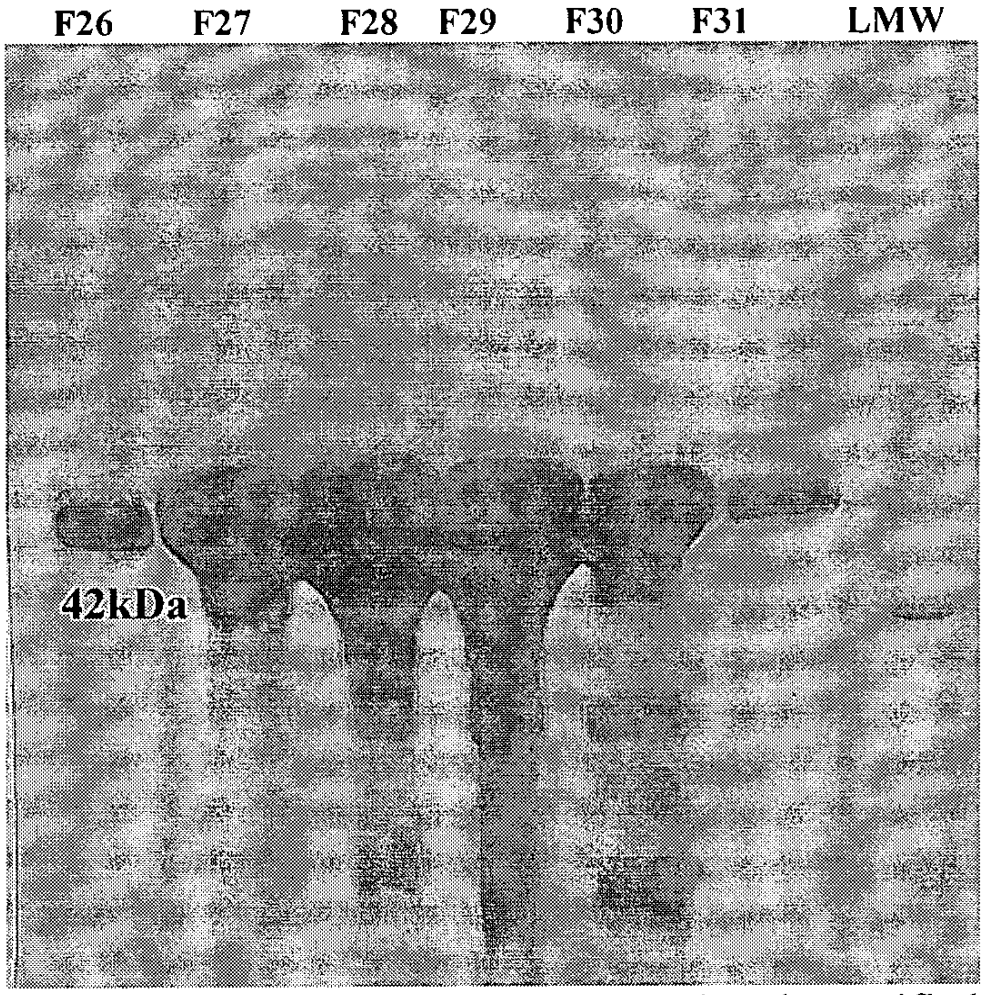


Figure 33. 12% SDS PAGE gel showing the purified E2 protein: Under denative conditions

LMW –Low Molecular Weight Marker, F26 - F31 protein fractions

Concentration of E2 protein 4.28 mg/ml

Preparation of CHIK proteins in yeast vector system- *P. pastoris*

Purification of CHIK proteins

E1 protein has a molecular weight of 45 kDa and pI of 6.4. E2 protein has a molecular weight of 42 kDa and pI of 7.4.

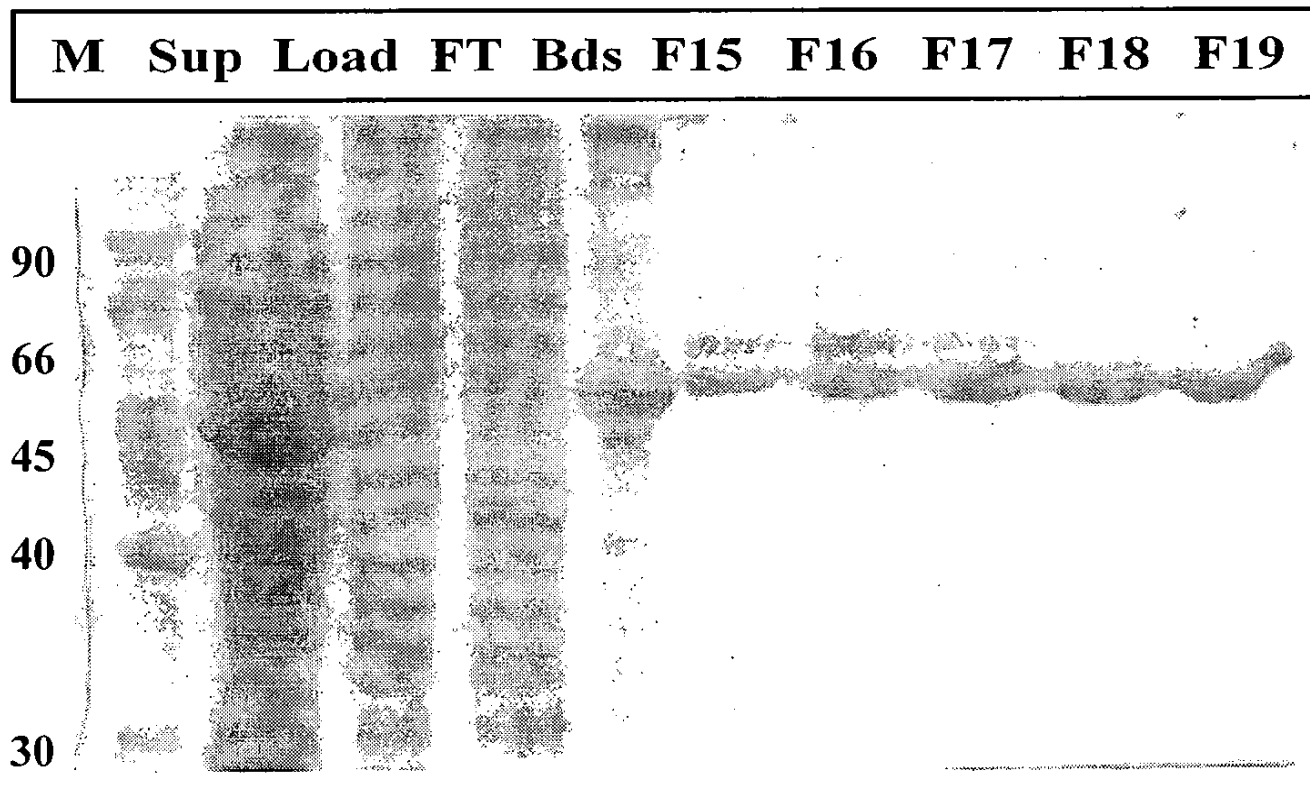


Figure 34.SDS-PAGE analysis of Ni-NTA affinity column fractions obtained during the purification of chikungunya E1 protein.

M- Low Molecular weight marker, Sup- Supernatant of the harvested *Pichia* culture, Load-Protein solution bound to Ni-NTA, FT-Flowthrough containing unbound proteins, Bds- Ni-NTA beads, F15-19- Eluted protein fractions

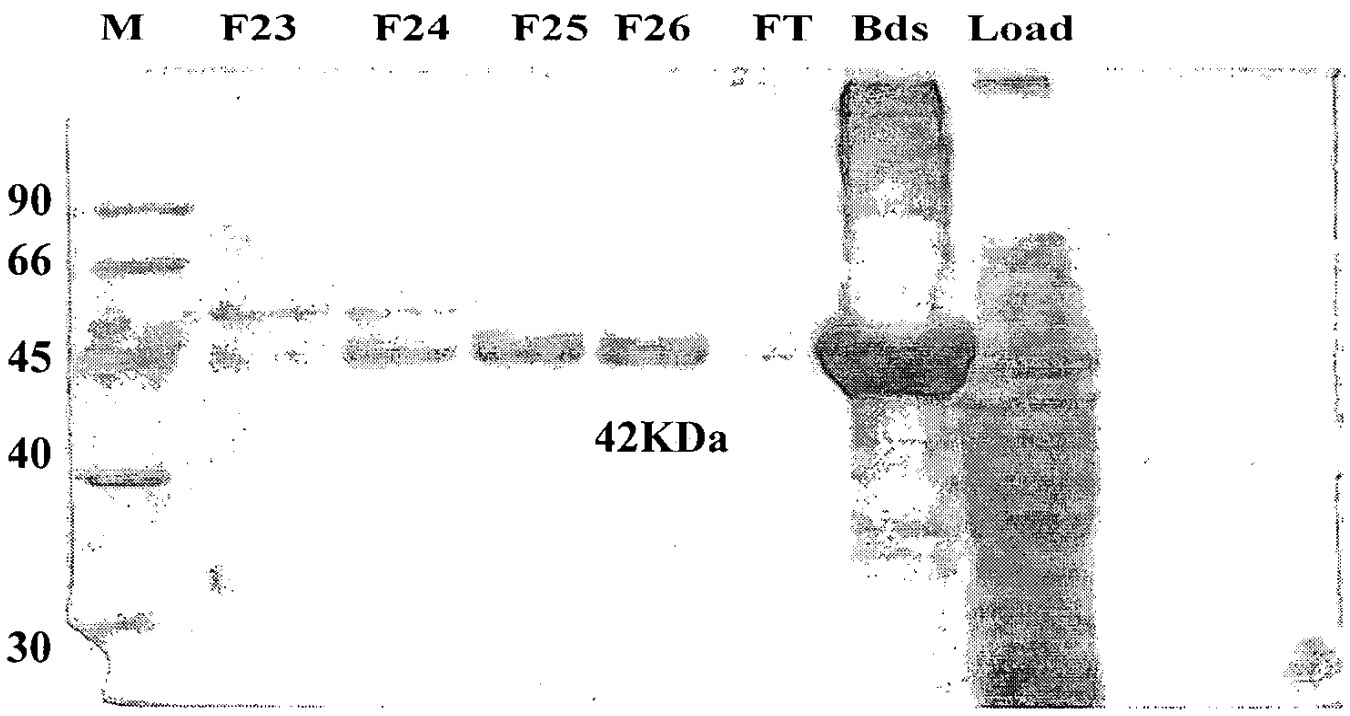


Figure 35. SDS-PAGE analysis of Ni-NTA affinity column fractions obtained during the purification of chikungunya E2 protein.

M- Low Molecular weight marker, F23-26- Eluted protein fractions, FT- flowthrough containing unbound proteins, Bds- Ni-NTA beads, Load-Protein solution bound to Ni-NTA

Development and optimisation of ELISAs using recombinant proteins expressed in *E. coli and P. pastoris*

Optimal assay conditions for IgM ELISA

- Coating 96 well flat bottom ELISA plates with 100 µl of diluted E1 and E2 protein (10 µg/ml in 0.1 M carbonate buffer pH 9.5) and incubate 4oC overnight , Washing with 1X PBS – 1 time.
- Blocking with 300 µl of blockase (5% skimmed milk, 2% PVP in 1 X phosphate buffered saline) at 37°C for 2 hours , Washing with a washing buffer (0.5% tween 20 in 1X PBS)- 2 times.
- Incubating with 100 µl of patient serum (diluted 1: 200 in sera diluent- 2.5% skimmed milk, 2% PVP, 25% Goat sera, 0.5 M KCl. 0.1% tween 20) at 37oC for 1 h, Washing with washing buffer - 4 times.
- Incubating with 50 µl of anti-human IgM-HRPO conjugate (diluted 1: 5000 in sera diluents) at 37oC for 1 hour, Washing with washing buffer – 6 times.
- Incubating with 100 µl of TMB soluble substrate at 37°C for 10 minutes.
- Stopping the reaction by adding 100 µl of 1 N H_2SO_4.
- Measuring the optical density value of each sample at 450 nm with 650 nm as the reference wavelength using a microplate reader.

Optimal assay conditions IgG ELISA

- Coating wells with 100 µl of diluted E1 and E2 protein (10 µg/ml in 0.1 M carbonate buffer pH 9.5) at 4°C overnight, washing wells with 1X PBS- 1 time
- Blocking with 300 µl of blockase (150 mM NaCl, 2.5% Bovine Serum Albumin (BSA). 5% Sucrose, 25% Goat Sera in 0.6% bovine gamma globulin in 50 mM carbonate bicarbonate buffer pH 9.6) at 37oC for 2 hours, washing with the washing buffer (0.5 M KCl, 1% Tween 20 in 50 mM carbonate bicarbonate buffer pH 9.6) - X 2 times
- Incubating with 100 µl of patient serum (diluted 1: 200 in sera diluent-150 mM NaCl, 2.5% BSA. 5% Sucrose, 25% Goat Sera in

0.6% bovine gamma globulin, 0.1% tween 20, 0.1% triton X 100 in 50 mM carbonate bicarbonate buffer pH 9.6) at 37oC for 1 hour, washing with washing buffer –X 4 times

- Incubating with 100 µl of anti-human IgG-HRPO conjugate (diluted 1: 10 000 in sera diluent) at 37oC for 1hour, washing with washing buffer X 6 times
- Incubating with 100 µl of TMB soluble substrate at 37oC for 10 minutes
- Stopping the reaction by adding 100 µl of 1 N H2SO4
- Measuring the optical density value of each sample at 450 nm with 650 nm as the reference wavelength using a microplate reader.

Cut off values for ELISAs using recombinant proteins expressed in *E. coli and P. pastoris*

Table 7. Average cut off values for ELISAs

Cut off values	E1 protein *(E. coli/Pichia)* (n=100)	E2 protein *(E. coli/Pichia)* (n=100)
IgM ELISA	1.474493838	0.40208624
IgG ELISA	0.678869795	0.654105393

Field evaluation of ELISAs using recombinant proteins expressed in *E. coli and P. pastoris*

No. of convalescent samples collected = 163

No. of samples positive samples
by both HAI and IgM ELISA using cell lysate antigen = 81
by both HAI and IgG ELISA using cell lysate antigen = 30

No of samples negative
by both HAI and IgM ELISA using cell lysate antigen = 22
by both HAI and IgG ELISA using cell lysate antigen = 30

Table 8. Results of IgM ELISA using recombinant E1 and E2 proteins for field samples

Protein	Positive number (n=81)	Negative number (n=22)	Sensitivity	Specificity
E. coli E1	26	20	32%	91%
E. coli E2	41	18	51%	81%
P. pastoris E1	38	18	47%	81%
P. pastoris E2	54	16	**67%**	**73%**

Table 9. Comparison of two types of IgG ELISAs

- *E. coli* E1 protein vs purified cell lysate antigen

		Purified Ag.		
		Positive (>= 1: 3,000)	Negative (<1: 3,000)	Total
Recombinant Ags. E. coli E1	Positive (>= 1: 3,000)	19	20	39
	Negative (<1: 3,000)	11	10	21
	Total	30	30	60

Recombinant Ag Vial-1 (Sensitivity : 63.3%, Specificity : 33.3%)

Table 10. Comparison of two types of IgG ELISAs

-*E. coli* E2 protein vs purified cell lysate antigen

		Purified Ag.		
		Positive (>= 1: 3,000)	Negative (<1: 3,000)	Total
Recombinant Ag. *E. coli* E2	Positive (>= 1: 7,000)	25	5	30
	Negative (<1: 7,000)	5	25	30
	Total	30	30	60

Recombinant Ag Vial-2 (Sensitivity : 83.3%, Specificity : 83.3%)

There is no significant difference between IgG ELISAs performed using

E2 proteins and cell lysate antigens (P=0.00, χ^2=24).

There is no significant difference between IgG ELISAs performed using E2 proteins and cell lysate antigens (P=0.00, χ^2=24).

Table 11. Comparison of two types of IgG ELISAs

-*P. pastoris* E1 protein vs purified antigen

		Purified Ag.		
		Positive (>= 1: 3,000)	Negative (<1: 3,000)	Total
Recombinant Ags. *P. pastoris* E1	Positive (>= 1: 3,000)	30	30	60
	Negative (<1: 3,000)	0	0	0
	Total	30	30	60

Recombinant Ag Vial-3 (Sensitivity : 100%, Specificity : 0.0%)

Table 12. Comparison of two types of IgG ELISAs

- *P. pastoris* E2 protein vs purified antigen

		Purified Ag.		
		Positive (>= 1: 3,000)	Negative (<1: 3,000)	Total
Recombinant Ag. *P. pastoris* E2	Positive **(>= 1: 10,000)**	27	2	29
	Negative **(<1: 10,000)**	3	28	31
	Total	30	30	60

Recombinant Ag Vial-4 (Sensitivity : 90%, Specificity : 93.3%)

There is no significant difference between IgG ELISAs between E2 protein and cell lysate antigens (P=0.00, χ^2 =38).

For Leptospirosis

Purification of leptospirosis proteins

Purification of leptospirosis proteins under denature condition in 6 M GuHCL

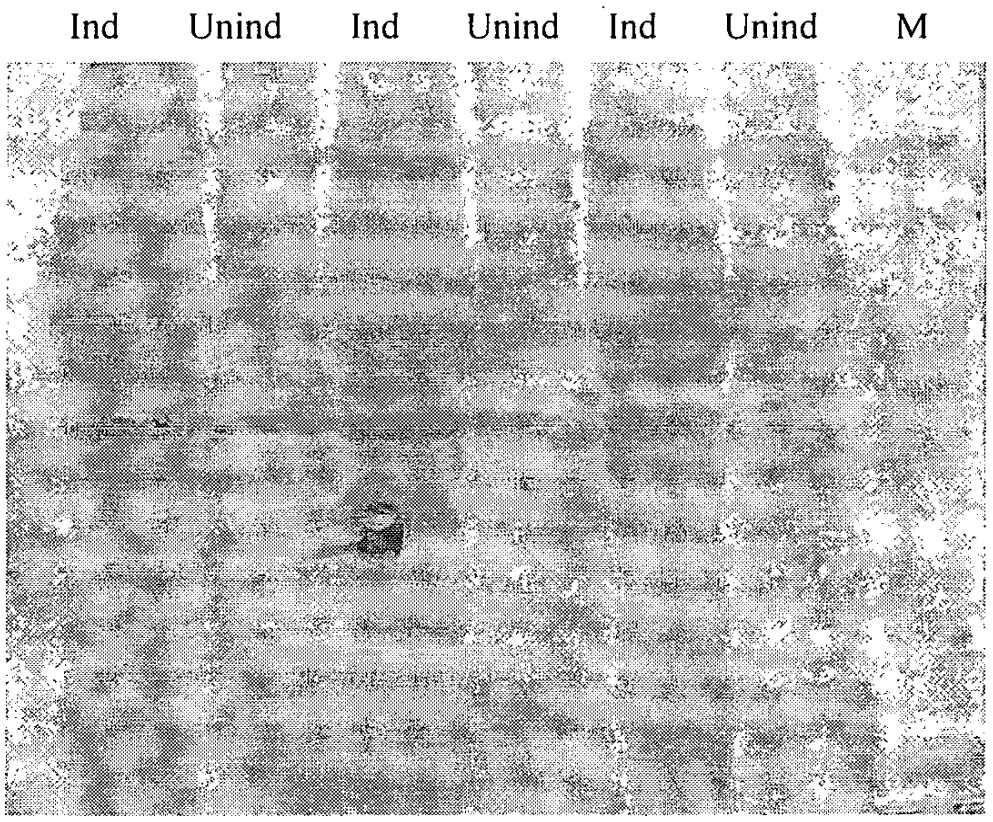


Figure 36.12% SDS PAGE gel showing the induction of Lipl32 protein

LMW –Low Molecular Weight Marker, Ind- Induced. Unind- uniduced

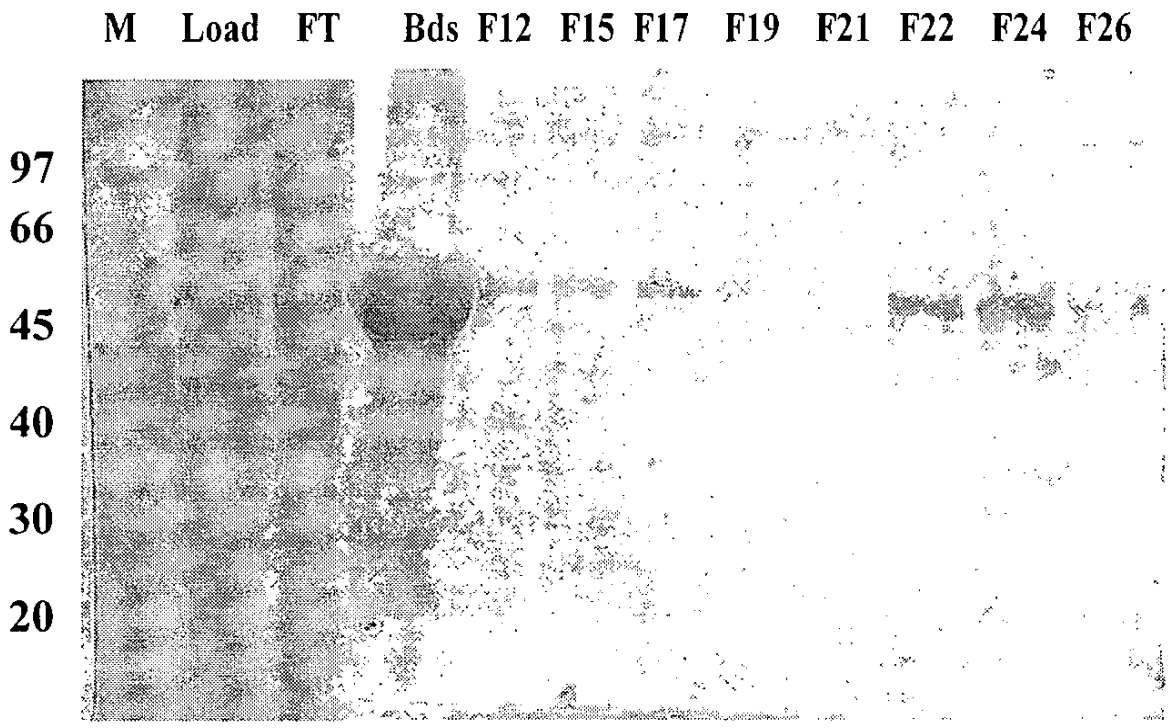


Figure 37.12% SDS PAGE gel showing the purified Lipl32 protein

LMW –Low Molecular Weight Marker, Bds- Beads, FT- Flow Through . F12 – F 26 protein fractions

Purification under denature condition in 6M GuHCL -Lipl 48 Protein

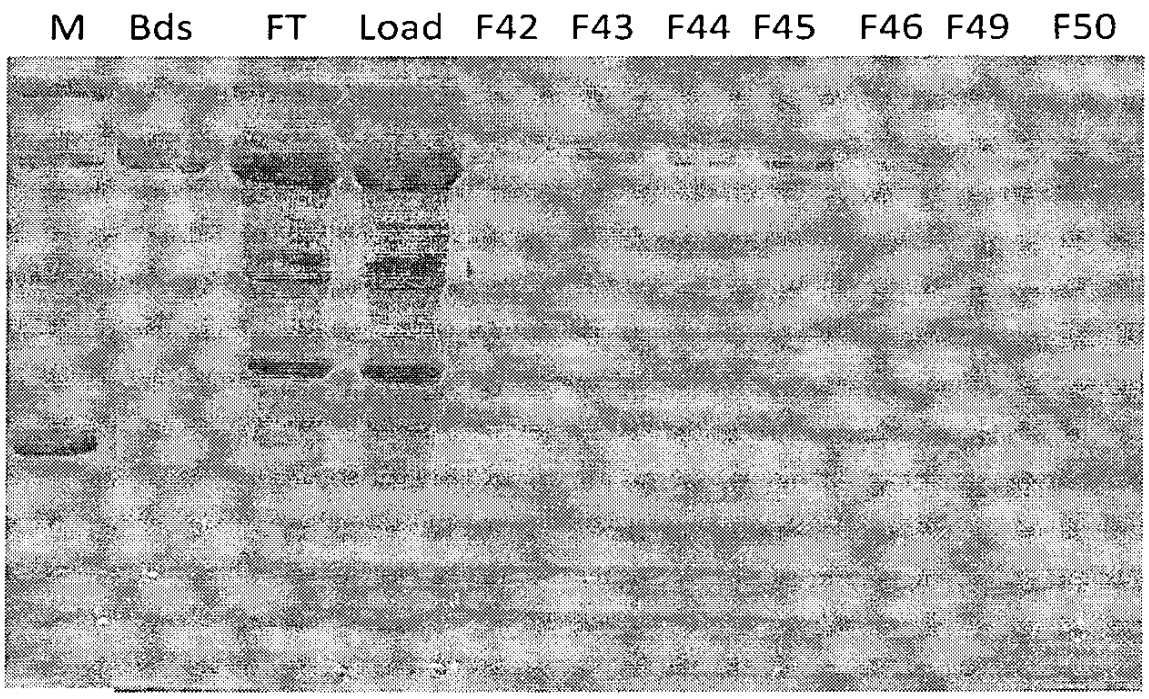


Figure 38. 12% SDS PAGE gel showing the purified Lipl32 protein:

LMW –Low Molecular Weight Marker, Bds- Beads, FT- Flow Through . F42 – F50 protein fractions

Optimal assay conditions for IgM ELISA

- Ninety six well flat bottom ELISA plates were coated with 100 µl of diluted Lipl32 protein (10 µg/ml in 0.1 M carbonate buffer pH 9.5) and incubated 4°C overnight.
- Washed with 1X PBS
- The wells were blocked with 300 µl of 5% skim milk, in 1 X phosphate buffered saline at 37°C for 2 hours
- Washed 2 X with 0.1% tween 20 in 1X PBS
- Washed wells were incubated with 100 µl of patient serum (diluted 1: 200 in sera diluent-2.5% skimmed milk, 0.1% tween 20) at 37°C for 1 h.
- wells were washed again four times with washing buffer
- incubated with 50 µl of anti-human IgM-HRPO conjugate (diluted 1: 5000 in sera diluents) at 37°C for 1 hour.
- wells were washed again four times with washing buffer
- incubated with 100 µl of TMB soluble substrate at 37°C for 10 minutes.
- The reaction was stopped by adding 100 µl of 1 N H_2SO_4.
- The optical density value of each sample was measured at 450 nm with 650 nm as the reference wavelength using a microplate reader.

Cut off values for ELISAs using recombinant proteins expressed in *E. coli* -Lipl 32

Table 13. Cut off values for Lipl 32

Cut off values	Lipl 32 protein
IgM ELISA	O.565

Characterization of acute and convalescent leptospirosis serum samples using available diagnostic assays

Table 14. Characterization of acute and convalescent leptospirosis serum samples

Type of sample	Type of assay	No. of positives
Acute (n=150)	LAMP (+)	87% (131/150)
	PCR-AGE (+)	19% (29/150)
Convalescent (n=106)	IgM ELISA-rapid assay (+)	49% (52/106)
	MAT (+)	34% (36/106)

Field evaluation of ELISAs using recombinant protein of leptospirosis

Table 15. Results of Lipl32 antigen for IgM and IgG ELISAs

Type of assay	No. of positive samples Positive number with
IgM ELISA-Lipl32 protein	78% (83/106)
IgG ELISA-Lipl32 protein	85% (90/106)

Table 16. Comparison of results of IgM ELISA using Lipl 32 antigen vs available diagnostic assays (either IgM ELISA rapid assay/MAT)

		IgM/MAT assay		
		Positive	Negative	Total
IgM ELISA Lipl32	Positive	54	29	83
	Negative	4	19	23
	Total	58	48	106

Recombinant Ag Vial-4 (Sensitivity : 93%, Specificity : 39%)

Table 17. Comparison of results of IgG ELISA using Lipl 32 antigen vs available diagnostic assays

		MAT assay		
		Positive	Negative	Total
IgG ELISA Lipl32	Positive	36	54	90
	Negative	0	16	16
	Total	36	70	106

Recombinant Ag Vial-4 (Sensitivity : 100%, Specificity : 23%)

Dengue

Characterization of acute and convalescent dengue serum samples using available diagnostic assays

Table 18. Characterization of acute and convalescent dengue serum samples using available diagnostic assays

Type of sample	Type of assay	Negative	Positive
Acute samples	PCR	61% (126/206)	39% (80/206)
Convalescent/follow-up sample 1 (7 days apart of acute sample)	IgM	68% (134/196)	32% (62/196)
	IgG	53% (103/196)	47% (93/196)
Convalescent/follow-up sample 2 (14 days apart of acute sample)	IgM	83% (63/76)	17% (13/76)
	IgG	79% (60/76)	21% (16/76)
	HAI	78% (59/76)	22% (17/76)

Field evaluation of IgM and IgG ELISAs (J.Mitra) vs available diagnostic assays

Table 19. Comparison results of IgM ELISA-J.Mitra vs IgM ELISA-Pan Bio on acute serum samples

		IgM ELISA-Pan Bio		
		No of Positive	No of Negative	Total
IgM ELISA J. Mitra	No of positive	61	2	63
	No. of negative	56	54	110
	Total	117	56	173

Recombinant Ag Vial-4 (Sensitivity : 52%, Specificity : 96%)

Table 20. Comparison results of IgM ELISA-J.Mitra vs IgM ELISA-Pan Bio and HAI on convalescent serum samples

		HAI/IgM ELISA-Pan Bio		
		No of Positive	No of Negative	Total
IgM ELISA J. Mitra	No of positive	39	-	39
	No of negative	23	14	37
	Total	62	14	76

Recombinant Ag Vial-4 (Sensitivity : 63%, Specificity : 100%)

Table 21. Comparison results of IgG ELISA-J. Mitra vs IgM ELISA-Pan Bio and HAI on convalescent serum samples

		HAI/IgG ELISA-Pan Bio		
		No of Positive	No of Negative	Total
IgG ELISA J. Mitra	No of Positive	56	-	56
	No of Negative	3	15	18
	Total	59	15	74

Recombinant Ag Vial-4 (Sensitivity : 95%, Specificity : 100%)

V. Discussion

For Chikungunya

Preparation and purification of recombinant protein antigens in *E. coli*

Finding parameters of each protein were important for purification of proteins. Isoelectric point (pI) is useful for preparing lysis buffer without precipitating the resulted recombinant protein.

E2 protein was expressed well with every plasmid in bacteria. But E1 protein was not expressed in bacteria with any of the plasmids. But when checked with western blot it has given a minute expression levels. E1 protein, when expressed in bacteria, could be toxic to itself. Therefore bacteria might be inhibiting or lowering the production of protein.

E1 and E2 protein in both pET32 and pET28 were not soluble in supernatant here proteins were in inclusion bodies. Purification under native condition was unsuccessful. This could be due to the highly insoluble nature of proteins. The protein could be in inclusion bodies.

Preparation and purification of recombinant protein antigens in *P. pastoris*

Inorder to increase solubility and expression proteins were expressed in *P. pastoris*. Here, both E1 and E2 proteins were expressed well in *P. pastoris* but only E2 expressed efficiently in *E. coli*. Resulted proteins in P.

pastoris have a high level of expression, it means production of high yield was achieved. Although both proteins were expressed well in this yeast system both proteins were not soluble, they were in the inclusion bodies.

Development of indirect IgM and IgG ELISAs using recombinant protein antigens to detect anti-CHIK antibodies

Optimal assay conditions for IgM and IgG ELISAs were developed by changing assay conditions using a checkerboard titration.

When consider the average cut off values of these two proteins E1 and E2 in *E. coli* and *P. pastoris,* only E1 gave considerably very high cut off value regardless of host. This could be due to low specificity of E1 protein antigen to detect CHIK antibodies but detecting other antigens produced by closely related organisms also.

At the late stage of the development of ELISAs, dialyzed E1 and E2 proteins in both hosts were used to increase sensitivity of detection. The dialysed both proteins did not show high absorbance compared to denatured protein. This could be due to misfolding of protein or the epitopes which recognizes the antibodies could be hidden inside the protein (not exposed to outside) due to refolding.

Analysis of specificity, sensitivity and agreement of IgM and IgG ELISAs performed using each protein antigen with serological assays

E2 protein in both hosts gave better sensitivity and specificity for detection of anti-CHIK antibodies used in this study.

E2 protein in *E. coli* has shown 51% of sensitivity and 81% of specificity in IgM ELISA. Further, the same protein has shown 83% of sensitivity and specificity in IgG ELISA when results were compared with standard methods.

E2 protein in *P. pastoris* has shown 67% of sensitivity and 73% of specificity in IgM ELISA. Further, the same protein has shown 90% of sensitivity and 93.3% specificity in IgG ELISA when results were compared with standard methods.

There is no significant difference between IgG ELISAs performed using E2 proteins in *E. coli* and cell lysate antigens (P=0.00, χ^2=24). Further, IgG ELISAs performed using E2 proteins in *P. pastoris* also showed the same results, having no significan difference with cell lysate antigens (P=0.00, χ^2 =38). Although there were no significant differences, E2 in both host systems showed high sensitivity and specificity.

This discrepancy in results would be due to lacks post translational modifications, protein folding (formation of Disulphide bonds between peptides) in *E. coli* expression system. Therefore, proper folding of protein does not occur after expression. Due to this reason the expressed recombinant proteins do not function as natural.

The difference between E1 and E2 proteins shown in this study might be explained by the fact that E1 protein is buried almost in the virus structure, which may give E1 protein less chance to exposure to host immune system. Analysing all the data, the protein that can be used as a diagnostic intermediate is E2 protein.

Selection of a single recombinant protein antigen as a diagnostic intermediate for IgM and IgG ELISAs

Considering these results E2 protein in *P. pastoris* has performed well in detecting both anti-CHIK IgM and IgG antibodies. This E2 protein in *P. pastoris* will be further studied and used as a diagnostic intermediate in the future.

For leptospirosis

Preparation and purification of recombinant protein antigens

Two proteins, Lipl32 and Lip48 proteins were expressed in a bacterial host system But only Lip32 protein was expressed. These proteins were not soluble in supernatant here proteins were in inclusion bodies. Purification under native condition was unsuccessful. This could be due to the highly insoluble nature of proteins. The protein could be in inclusion bodies.

Development of indirect IgM and IgG ELISAs using recombinant protein antigens to detect anti-leptospirosis antibodies-Lipl32 protein

Optimal assay conditions for IgM and IgG ELISAs by LIPl32 protein were developed by changing assay conditions using a checkerboard titration. When consider the cut off values of this Lipl32 protein, it gave considerably low cut off value.

Analysis of specificity and sensitivity IgM and IgG ELISAs performed using Lipl32 protein with serological assays

IgM ELISA using Lipl32 protein gave better sensitivity (93%) low specificity (39%) for detection of anti-leptospirosis IgM antibodies when compared with standard methods (MAT and IgM rapid ELISA). There is no significant difference between IgM ELISAs using Lipl32 with MAT/IgM rapid ELISA ($P=0.00$, $\chi^2=14$).

IgG ELISA using Lipl32 protein gave better sensitivity (100%) low specificity (23%) for detection of anti-leptospirosis IgG antibodies when compared with MAT. There is no significant difference between IgG

ELISAs using Lipl32 with MAT (P=0.004, χ^2=8).

Selection of a single recombinant protein antigen as a diagnostic intermediate for IgM and IgG ELISAs

IgM and IgG ELISAs for leptospirosis can be done using Lipl32 protein but further modifications should be done to reduce background

- by dialysing of protein
- by changing buffer conditions

For Dengue

Field evaluation of IgM ELISA (J.Mitra)

IgM ELISA kits given by J.Mitra Company were used on acute serum samples. These acute serum samples gave better sensitivity (52%) and high specificity (96%) for detection of anti-dengue IgM antibodies when compared with standard methods (HAI and IgM ELISA). There is no significant difference between IgM ELISAs-J.Mitra with current assays on acute serum samples (P=0.00, χ^2=36).

IgM ELISA kits given by J.Mitra Company were used on convalescent serum samples. These convalescent serum samples gave better sensitivity (63%) and high specificity (100%) for detection of anti-dengue IgM antibodies when compared with standard methods (HAI and IgM ELISA). There is no significant difference between IgM ELISAs-J.Mitra with current assays on acute serum samples (P=0.00, χ^2=15).

IgM ELISA kits given by J.Mitra Company were used on convalescent serum samples. These convalescent serum samples gave better sensitivity (63%) and high specificity (100%) for detection of anti-dengue IgM antibodies when compared with standard methods (HAI and IgM ELISA). There is no significant difference between IgM ELISAs-J.Mitra with current assays on acute serum samples (P=0.00, χ^2=15).

Field evaluation of IgG ELISA (J.Mitra)

IgG ELISA kits given by J.Mitra Company were used on convalescent serum samples. These convalescent serum samples gave better sensitivity (95%) and specificity (100%) for detection of anti-dengue IgM antibodies

when compared with standard methods (HAI and IgM ELISA). There is no significant difference between IgM ELISAs-J.Mitra with current assays on acute serum samples (P=0.00, χ^2=53).

Vi. Conclusions

The resulted proteins which show the highest sensitivity and specificity with currently available diagnostic assay were selected. Resulted proteins are available at two counterpart institutions for field use and further studies. Target group beneficiaries at the end of the project will be the infected people and healthy persons in outbreak area/s of these three diseases by confirming each disease using ELISAs developed using these recombinant protein antigens. This type of study on development of diagnostic intermediates have a significant effect for rapid confirmation of outbreaks and limit spread of such outbreaks from one geographical area to another. Further, confirmation of disease outbreaks will be avoided loss of working hours and socio-economical impact on individuals and government.

In order to synergize activities, the work was carried out jointly by the Molecular Medicine Unit, Faculty of Medicine, University of Kelaniya, Sri Lanka (main counterpart institution) and Mammalian Biology-Recombinant Gene Products (RGP) Group, International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi resident. Here these two institutions worked with each other through exchanging knowledge and resources (human and technical). Main counterpart institution worked together with clinicians, general practitioners and epidemiologist in notifying and locating cases at the regional level when they collected clinical samples from suspected patients of each disease. Further, main counterpart institution collaborated with national and international reference centers to characterize three panels of serum samples by the Gold standard assay for each disease. Further, during the operation period, suspected patients for each disease warded in hospital/s in outbreak area/s in Sri Lanka were benefited by having results of laboratory diagnostic assays (molecular and currently available serological assays) within 48 hours of collection of the sample. Funds amounting EURO 36,000.00 received for the main counterpart institution through the project were used to meet the cost of equipment, consumables, training and travel. The main counterpart institution has set up facilities for molecular diagnosis and preparation of recombinant proteins for each disease. One under graduate and three post graduate students were directly benefited by the project. Results of the project were disseminated through number of presentations made at national and international conferences

and a post graduate thesis. Further, dissemination of project results will be made through journal publications in the future.

vii. References

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viii. Problems if any, encountered during the implementation of the project

No

ix. Major findings and follow up activities

Major findings-

- Recombinant protein antigens which can be used as diagnostic intermediates for indirect ELISAs for CHIK, dengue and leptospirosis
- Two Ph.D. thesis, number of conference proceedings
- Networking few instutions

Follow up activities

- Publishing journal articles and commercializing diagnostic kits

SECTION 4.

IMPACT OF RESEARCH RESULTS

i. Relevance of results achieved to scientific advancement

Development and evaluation of diagnostic capability of novel recombinant protein antigens for CHIK, dengue and leptoswere these proteins performed in the study. Resulted proteins are available at two counterpart institutions for field use and further studies.

ii. Relevance of results achieved to national/socio-economic development

Target group beneficiaries at the end of the project will be the infected people and healthy persons in outbreak area/s of these three diseases by confirming each disease using ELISAs developed using these recombinant protein antigens. This type of study on development of diagnostic intermediates have a significant effect for rapid confirmation of outbreaks and limit spread of such outbreaks from one geographical area to another. Further, confirmation of disease outbreaks will be avoided loss of working hours and socio-economical impact on individuals and government.

iii. Dissemination/application of research out put

Provide project results to health authority for patient management: Results of the molecular-based diagnostic assay and commercial IgM and IgG ELISAs (dengue and leptospirosis) were sent to the inpatient wards/health authority of the relevant hospital/s in the outbreak area within 48 hours.

Write- up reports, research papers and thesis: Project progress reports were submitted yearly. Writing research papers based on results of the project is on going. Acknowledgement will be given to the grant and a copy of the paper will be sent once they will be published. Research Assistant, Miss. Maheshi Athapaththu submitted her Ph.D. thesis titled "Development of recombinant protein antigens as diagnostic intermediates for Chikugunya" with the acknowledgment to the NSF (Annexure 4). She has obtained her degree in December, 2013.

Present project findings in national/international conferences: Results of the project were disseminated through number of presentations made at national and international conferences and a post graduate thesis.

SECTION 5.

MISCELLANEOUS

i. List of major equipment acquired during the project period and their functionality

Not relevant

ii. List of publications/communications arising from the project and/or presentations made at seminars, workshops etc (Please attaché copies).

Conference proceedings –International

1. Athapaththu, A. M. M. H., Khanna, N., Abeyewickreme, W., Gunasena, S. and Hapugoda, M. (2010). Enzyme-Linked Immunosorbent Assay (ELISA) using recombinant protein antigens for detection of anti-chikungunya antibodies. *Proceedings of the Joint International Tropical Medicine Meeting*, Bangkok 103.

Conference proceedings -National

1. Athapaththu, A. M. M. H., Khanna, N., Gunasena, S., Abeyewickreme, W. and Hapugoda, M. (2010). Development of recombinant proteins as diagnostic intermediates for chikungunya infection. *Proceedings of the Sri Lanka Association for the Advancement of Science* **65:** 10.

2. Athapaththu, A. M. M. H., Khanna, N., Inouve, S., Tun, M. M. N., Gunasena, S., Abeyewickreme, W. and Hapugoda, M. (2011). Comparison of recombinant protein and cell lysate antigens for detection of anti-chikunungya IgM antibody. *Proceedings of the Sri Lanka College of Microbiologists.* **09:** 16 *(This abstract has been awarded the second place in oral presentation by the College of Microbiologist).*

3. Athapaththu, A. M. M. H., Khanna, N., Inouve, S., Tun, M.M.N., Gunasena, S., Abeyewickreme, W. and Hapugoda, M. (2013). Development of recombinant protein antigens using a bacterial expression system for the detection of anti-Chikungunya (CHIK)

antibodies. *Proceedings of the Sri Lanka College of Microbiologists.* **11 (1):** 14 *(This abstract has been awarded the second place in oral presentation by the College of Microbiologist).*

4. Athapaththu, A.M.M.H., Khanna, N., Inouve, S., Gunasena, S., Abeyewickreme W. and Hapugoda, M. (2013). Comparison of recombinant Chikungunya (CHIK) E2 antigens expressed in bacterial and eukaryotic systems for the detection of anti-CHIK antibodies in human serums samples. *Proceedings of the 14th of Annual Research Symposium, Faculty of Graduate Studies, University of Kelaniya* 68-69.

5. Athapaththu, A.M.M.H., Khanna, N., Inouve, S., Gunasena, S., Abeyewickreme W. and Hapugoda, M. (2013). Development of recombinant protein antigens using a yeast expression system, for the detection of anti-Chikungunya (CHIK) antibodies in clinical samples. *Proceeding of the Annual scientific sessions Sri Lanka Association for the Advanced of Science (SLAAS)* 12.

6. Denipitiya, D.T.H., Chandrasekharan, N.V., Abeyewickreme, W. and Hapugoda, M.D. (2013). Detection of pathogenic *Leptospira* in rat blood samples by molecular-based assays. *Proceedings of the 14th of Annual Research Symposium, Faculty of Graduate Studies, University of Kelaniya* 72-73.

7. Denipitiya, D.T.H., Athapaththu, M., Chandrasekharan, N.V., Abeyewickreme, W. and Hapugoda M.D.(2013). Risk factors associated with human leptospirosis in the District of Gampaha, Sri Lanka. *Proceedings of the 14th of Annual Research Symposium, Faculty of Graduate Studies, University of Kelaniya* 73-74.

8. Denipitiya, D.T.H., Jiffriy, A.M., Chandrasekharan, N.V., Abeyewickreme, W. and Hapugoda, M.D. (2013). Evaluation of a real time Polymerase Chain Reaction (PCR) assay for the early diagnosis of human leptospirosis. *Proceeding of the Annual scientific sessions Sri Lanka Association for the Advanced of Science (SLASS)* 21.

9. Denipitiya, D.T.H., Chandrasekharan, N.V., Abeyewickreme, W. and Hapugoda, M.D. (2013). Evaluation of a case definition for leptospirosis diagnosis using serological assays. *Proceedings of the Annual scientific sessions, Sri Lanka Association for the Advanced of Science (SLAAS)* 20.

10. Denipitiya, D.T.H., Jiffriy, A.M., Chandrasekharan, N.V., Abeyewickreme, W. and Hapugoda, M.D. (2014). A comparison of three molecular-based assays to detect pathogenic leptospires in cattle urine. *Proceedings of Peradeniya University International Research Session* **18:** 378.

Research papers-Under preparation

SECTION 6.

SUMMARY STATEMENT OF EXPENDITURE

SECTION 7.

SIGNATURES

i. **Grantees signatures**

ii. **Comments of the Head of the Department/Dean of the Faculty**

Recommended

Dean
Faculty of Medicine
University of Kelaniya
Sri Lanka

iii. **Head of the institution's signature**

Actg. **VICE-CHANCELLOR**
University of Kelaniya
Kelaniya, SRI LANKA.

ANNEXURE I. Questionnaire used for patient information

ANNEXURE 1A. CHIKUNGUNYA

UNIVERSITY OF KELANIYA, SRI LANKA
FACULTY OF MEDICINE
P.O. Box 6, Thalagolla Road, Ragama, Sri Lanka.
Molecular Medicine Unit *Tel-0112-960483* *Fax: 0112-2953412*

PATIENT INFORMATION- Chikungunya

PROJECT TITLE: Development of recombinant proteins as diagnostic intermediates for chikungunya

Inclusion criteria: Fever $\leq$ 5 days
Arthralgia
Myalgia

Exclusion criteria: Diagnosed Arthritis or connective tissue disorder
Severe Anaemia
Bleeding disorder

Interview date:
Patients' ID code:
Age:
Sex: Male ☐ Female ☐

Race: Sinhala ☐ Tamil ☐ Muslim ☐ Other ☐

Occupation:

Personal address:

Contact no.:
Map of location/route:

GN division:
MOH division:

Clinical data:
Name of hospital:
Ward no:
BHT:
Date of admission:
Fever days: Highest recorded ……F/C on …….day

Other associated symptoms:

	Absent	Present		
		mild	moderate	severe
Headache				
Backache				
Nausea				
Vomiting				
Facial swelling				
Photophobia				
Arthralgia				
Myalgia				
Skin rash				
Joint pain and swelling:				

Type of joint	Pain		Swelling	
	Y/N	Description	Y/N	Description
Knee joint				
Ankle joint				
Wrist joint				
Elbow joint				
Small joints				
Other				

No of days absent from work:
Previous CHIK attacks: Y ☐ N ☐
When:
Where:
Previous dengue attacks: Y ☐ N ☐
When:
Where:
Did you visit CHIK areas recently? Y☐ N ☐

Sample collection:

	Date and time	Blood volume (5 ml)	Date of transport to MMU
Acute D0			
Convalescent/follow up 1			
Convalescent/follow up 2			

Laboratory investigations:

WBC.....mm^3 , DC: N.....% L.....%

Platelet count: D1.....D2.....D3.....

Hb% & PCV: D1.....D2.....D3.....

Max SGPT:.....on.....day; return to normal on .....day

Chikungunya RT-PCR-AGE assay: .....
Chikungunya antibodies: IgM.....
IgG.....

ANNEXURE 1B. Leptospirosis

UNIVERSITY OF KELANIYA, SRI LANKA

FACULTY OF MEDICINE
P.O. Box 6, Thalagolla Road, Ragama, Sri Lanka.

Molecular Medicine Unit Tel-0112-960483 Fax: 0112-2953412

PATIENT INFORMATION- Leptospirosis

PROJECT TITLE: Development of recombinant proteins as diagnostic intermediates for chikungunya

Inclusion criteria: Acute febrile illness with headache, myalgia and prostration, fever 1 - 5 days (According to the tentative diagnosis made by physician on admission)

Exclusion criteria: Patients who is critically ill and can not provide the history, Patients who have treated with doxycyline and peniciline derivatives.

Interview date: .....Patients ID code:
.....

A. PARTICULARS OF PATIENTS

Name and address :
.....

Patient's contact number : .....
Age / Date of Birth : ..... Sex:
.....
Ethnic group: ..... Name of Hospital:
.....
Ward No: .... Consultant physician:
.....
BHT: .....

B. SAMPLE COLLECTION

Onset of fever	Date and time	Blood volume (5ml)	Date of Transport to MMU
D.....			
D.....			

C. PRESENT ILLNESS / OUTCOME

i. Date of admission: .....
ii. Date of Onset of symptoms: .....

iii. Fever days: ..... Highest recorded ..... F/C on ..... day
iv. Out come of the case : cured / transferred / died / not known
v. Date of Discharge / transfer / died: .....
vi. Is the patient on antibiotic treatment

D. CLINICAL DATA

Clinical Details

Flu – like illness ☐
Pyrexia
Headache ☐
Myelgia ☐
Vomiting
Diarrhea ☐
Conjunctivitis ☐
Jaundice
Meningeal Irritation ☐
Anuria/oliguria ☐
Haemorrhage
Cardiac failure Arrhythmic ☐
Skin rash
Any other

E. INFORMATION ON DISEASE TRANSMISSION

Possible Source of Contamination ☐
Paddy field
☐ Other Agricultural Land (Sugar cane, Chena)
☐ Marshy / Muddy land
☐ Other Water related Source (Sewer, irrigate)
Animal husbandry, Veterinary fisheries
☐ Other (specify)

F. HISTORY OF RECENT SKIN LESION / INJUNY

G. Have you or your family members been infected with leptospirosis before?

Do you know anyone who had leptospirossis recently? Yes / No
If yes please specify ? (Neighbors, at working place, etc)
Did you visit a place where leptospirosis infection has been epidemic recently?

H. PROPHYLAXIS

Is the patient on prophylaxis treatment: .....

I. CLINICAL LABORATORY FINDINGS

Urine analysis WBC/DC

Albumin ……..
Pus cells ……..
Red cells ……..
Casts ……..

Total ……..
N ……..
L ……..
E ……..

Platelet count: ……..
Others (specify):
……..
Serum Bilirubin/ Direct/
Indirect

LABORATORY FINDINGS DIAGNOSIS

Test	1st Sample (Acute)	2nd sample (Con.)	Remarks
PCR – AGE			
ELISAs – Present Ig M Ig G			
LAMP Assay			

ANNEXURE 1C. Dengue

UNIVERSITY OF KELANIYA, SRI LANKA

FACULTY OF MEDICINE
P.O. Box 6, Thalagolla Road, Ragama, Sri Lanka.

Molecular Medicine Unit Tel-0112-960483 Fax: 0112-2953412

PATIENT INFORMATION- Dengue

PROJECT TITLE: Development of recombinant proteins as diagnostic intermediates for chikungunya

Part 1 Information of the patient

Ref. No .....
Date .....
Name &
address.....

.....
Ward .....BHT .....Date of admission.....Date of discharge.....
Fever days ..... Highest recorded .....F^0/C^0on .....day
Age..... Sex.....

Part 2 Clinical and laboratory information

Associated symptoms

Headache: Y/N; mild/moderate/severe. Onset:.....day,lasted.....days
Rectro-orbital pain; Y/N; mild/moderate/severe. Onset:.....day,lasted.....days
Neck pain: Y/N; mild/moderate/severe. Onset:.....day,lasted.....days
Limb pain: Y/N; mild/moderate/severe. Onset:.....day,lasted.....days

Bleeding manifestations: Y/N
If yes: nasal / gum / haematamesis / melaena / skin patches
Occurred on: ...../...../...../...../.....

Signs: Flushing/muscle tenderness/lymphadenopathy/palatal petechiae
Liver Y/N.....cm Spleen Y/N.....cm
Pulse...../min BP...../.....mmHg

Laboratory investigations:

WBC.....mm^3, DC: N...% L.....%
Platelet count: D1.....D2.....D3.....
Hb %& PCV: D1.....D2.....D3.....
Max SGPT:.....on.....day; return to normal on.....day

Annexure 2. Consent form

UNIVERSITY OF KELANIYA, SRI LANKA
FACULTY OF MEDICINE
P.O. Box 6, Thalagolla Road, Ragama, Sri Lanka.

Molecular Medicine Unit Tel-0112-960483 Fax: 0112-2953412

Research consent form for withdrawing blood (adult)

Patient's reference no:.....

Project title: Development of recombinant proteins as diagnostic intermediates for chikungunya, dengue and leptospirosis

Name of investigators: Dr. Menaka Hapugoda and Prof. W.Abeyewickreme

I, ... volunteer to participate in the study titled "Development of recombinant proteins as diagnostic intermediates for chikungunya, dengue and leptospirosis" as decided by the investigators and to donate 3 blood samples (5 ml/each sample) at first second and third visits to be used only for research purposes. The blood will be used to study on development of recombinant proteins as diagnostic intermediates for chikungunya, dengue and leptospirosis. The effects which are likely to occur during bleeding include mild pain, bruising and rarely fainting or mild infection at the punture site. An incentive will not be given to me for this purpose. I understand that my participation in this study will be strictly confidential.

.....Signature of the donar
.....Signature of the investigator/authorized officer
.....Signature and name of the witness
.... Date

Annexure 3. Work plan

	Months		
Activity	**1 - 3**	**4 - 33**	**34 - 39**
Pre-analytical phase (3 months), below activities were carried out. ➢ Conduct discussions between two counterpart institutions. ➢ Collect literature ➢ Conduct awareness programmes to clinicians and establish communication channels for obtaining outbreak epidemiological information. ➢ Establish a collaborative network with reference laboratories. ➢ Prepare data and sample collecting tools. ➢ Establish molecular and conventional diagnostic assays for each disease.			
Analytical phase (30 months), below activities were carried out. Phase 1. Prepare recombinant protein antigens to be used as diagnostic intermediates (RGP, ICGEB, New Delhi) ➢ Visit Principal Investigator to the collaborative institution- RGP, ICGEB, New Delhi ➢ Design recombinant protein antigens			

➢ Construct recombinant plasmids ➢ Clone, express and purify recombinant 6× His tagged proteins ➢ Characterize each protein using western blot analysis ➢ Assessment of reactivity of each protein towards antibodies using western blot analysis ➢ Develop and optimize inhouse ELISAs using recombinant proteins ➢ Train project personals <u>Phase 2. Prepare well characterized serum panels for each disease (University Kelaniya, Sri Lanka)</u> ➢ Obtain (ethical) permission ➢ Establish a research team from the existing resources ➢ Prepare a proper transportation system ➢ Prepare data/sample collecting tools ➢ Select sampling sites and human population ➢ Collect clinical, clinical laboratory information and exposure history from suspected patients ➢ Collect clinical samples from suspected patients			

➢ Confirm suspected patients for each disease by laboratory diagnostic assays Phase 3. Prepare serum panels to determine cut off values of ELISAs (University of Kelaniya, Sri Lanka) ➢ Collect serum samples from healthy volunteers ➢ Confirm healthy volunteers ➢ Determine the cut off OD value Phase 4. Evaluate recombinant protein antigens on clinical samples as diagnostic intermediates (University of Kelaniya, Sri Lanka and Nagasaki University, Japan)			
Post analytical phase (6 months), below activities were carried out Phase 5. Carry on common activities (University of Kelaniya, Sri Lanka) ➢ Enter and manage computer-aided data bases ➢ Analyze research data			

➢ Disseminate project results at national and international level <u>Phase 6. Provide routine laboratory diagnostic facilities to the general public (University of Kelaniya, Sri Lanka)</u> ➢ Prepare and implement a Standard Operational Procedures (SOP) and safety manual for each diagnostic test. ➢ Provide routine laboratory diagnostic facilities to the general public			

ANNEXURE 4.

Research publications/Communications

JITMM2010 & IMC2010

"Tropical Diseases: future threats and new paradigms"

and

International Malaria Colloquium 2010 (IMC2010)

"Malaria: new hopes, new challenges"

Abstracts

1-3 December 2010

Centara Grand & Bangkok Convention Centre at CentralWorld

Bangkok, Thailand

GENETIC POLYMORPHISM OF US28 OF HUMAN CYTOMEGALOVIRUS INFECTION IN HIV INFECTED AND NON HIV-INFECTED CHILDREN

Rujee Leecharoen, Assist. Prof.
Saowakon Paca-uccaralertkun

Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, 10400

Objectives - To study the polymorphism of US28 genes of HCMV infection in HIV infected and non HIV-infected children from Phayathai Babies's Home, Nonthaburi, Thailand

Methods – 43 HCMV positive urine samples (HIV=11samples, non HIV=32 samples) from Phayathai Babies's Home children with age less than 5 years old were used in this study. Viral DNA from urine sample was extracted and purified by Axygen® AxyPrep body fluid viral DNA/RNA miniprep kit. The nested polymerase chain reaction (nested-PCR) method was used to study the polymorphism on US28 gene N-terminus domain. The DNA sequencing determination was analyzed by sequencer software, MEGA 4.0 program then compared the homology of the sequences to the published sequences from GenBank. The variation of amino acid substitutions at the N-terminus domain of HIV and non HIV-infected children were compared. Relation of amino substitution and gB genotype was studied.

Result - Sequencing analysis of the US28 gene N-terminus domain reveals some amino acid substitutions: A8T, D15E, E18D/L, D19A/G/E, V24T, F25L, and L45I. Percent of amino acid substitution at the N-terminus domain of the US28 gene in non HIV-infected children is appeared to be higher than that of HIV infected children. Among gB genotypes, the variation of amino substitutions is highest in the gB1 genotype. The AD169 homologous sequence of the US28 gene N-terminus domain was observed more frequent in samples of HIV infected children. ❖

Keywords: HCMV, polymorphism, GPCRs, US28

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) USING RECOMBINANT PROTEIN ANTIGENS FOR DETECTION OF ANTI-CHIKUNGUNYA ANTIBODIES

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Objectives: Chikungunya is a mosquito-borne viral infection that has caused great medical and public health problems in South East Asia during last few years. Currently available laboratory diagnostic kits depend on Enzyme-Linked Immunosorbent Assay (ELISA) based on whole viral antigens caused biohazard risk, high production cost and cross reactivity with other organisms of the same genus/family. These problems can be avoided by using recombinant protein antigens in ELISAs.

Methodology: Two novel recombinant protein antigens based on Envelope (E) domain, a critical antigenic region of the major structural protein of chikungunya virus were expressed separately in a bacterial expression system (*Escherichia coli*). Two proteins were purified under denatured conditions. They were evaluated as potential diagnostic intermediates for detection of anti-chikungunya antibodies in Immunoglobulin M (IgM) and Immunoglobulin G (IgG) ELISAs separately using a panel of serum samples confirmed by the gold standard assay, Heamagglutination Inhibition (HAI) assay.

Results: These 2 protein antigens: E1 and E2 showed more than 60% positivity in IgG ELISAs and IgM ELISAs. A field validation using a large number of serum samples should be done for further confirmation of these results. It can be concluded that these 2 novel recombinant protein antigens can be used as a diagnostic intermediate to detect anti-chikungunya antibodies. ❖

Keywords: chikungunya, ELISA, recombinant proteins
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110/A

Development of recombinant proteins as diagnostic intermediates for chikungunya infection

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Chikungunya is an important disease with explosive outbreaks occurring in many South East Asian countries. As the clinical symptoms associated with chikungunya viral infections are often indistinguishable from those of many other viral, bacterial and parasitic infections confirmation of chikungunya outbreaks is important for clinicians for proper management of patients and for vector control programmers. Laboratory diagnosis of chikungunya in Sri Lanka is hindered by the non-availability of reliable commercial diagnostic kits and inaccessibility to reagents. There is a need to develop an assay that can confirm chikungunya, produced at low cost and easily standardized for the use in field settings. Currently available laboratory diagnostic kits depend on ELISA based on whole viral antigens which cause biohazard risk, high production cost and cross reactivity with other organisms of the same genus/family. Therefore, a diagnostic intermediate using a single recombinant protein antigen to overcome problems associated with whole viral antigen/lysate is important. The objective of this study was to assist laboratory confirmation of outbreaks through developing competencies for a rapid laboratory diagnostic method using recombinant protein antigens for chikungunya infection.

We have designed 2 novel recombinant protein antigens based on Envelope domain (E), a critical antigenic region of the major structural protein of chikungunya virus. They were expressed in *Escherichia coli* separately, and resultant proteins were affinity purified and obtained ~5mg and ~10mg respectively and protein of >95% purity per liter of culture. These 2 proteins were evaluated as potential diagnostic intermediates in ELISA separately for the detection of anti-chikungunya Immunoglobulin M (IgM) antibody using a panel of well characterized serum samples. E1 and E2 showed 60% and 67% positivity respectively. Specificity proteins were tested using serum from healthy volunteers and infected with other viral diseases. Two proteins could detect only anti-chikungunya IgM antibodies. We demonstrated that these 2 novel recombinant protein antigens can function as diagnostic intermediates.

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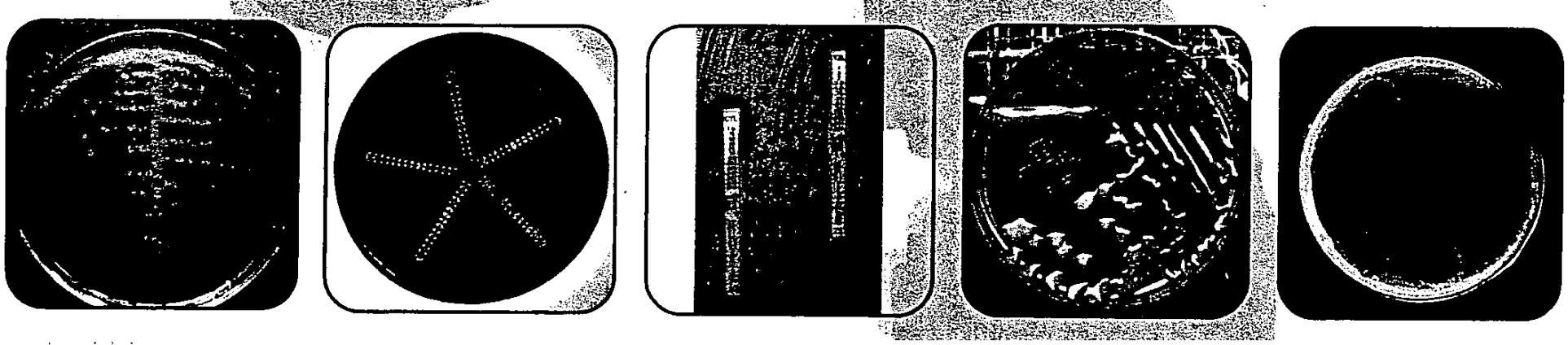

Stokes' method:
Chloramphenicol, amikacin and ceftazidime met the QC requirements of the Stokes' method. Gentamicin and cefuroxime had 2 and 3 of 30 readings respectively below the expected range.

Conclusion

Further work is needed before establishing the CLSI method in routine laboratories unable to perform daily QC on all antibiotics tested. The Stokes method may provide a more robust and flexible method for ABST in resource limited laboratories.

OP 5

Immunogenicity study to assess rabies neutralizing antibodies in previously immunized patients

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Introduction

A significant number of patients (11%) who seek anti rabies post exposure therapy (PET) following a re-exposure, had received either a full or the first three doses (partial course) of anti rabies vaccine (ARV) previously. There are no recommended guidelines on treatment of patients

Objectives

To assess rabies virus neutralizing antibody titre (RVNAT) in patients who received PET at different times previously and to assess the booster response following subsequent intradermal (ID) ARV therapy.

Methodology

Eighty two patients exposed to suspected rabid animals with a past history of ARV therapy were enrolled. Patients were categorized into four groups depending on duration following the last dose of previous ARV (Group - 1: <6 months; 2: 6 months - 2 years; 3: 2-5 years; 4: >5 years). RVNAT were determined by rapid fluorescent focus inhibition test on day 0 (D0) and day 14 (D14) post-vaccination following 2 or 3 ARV booster doses.

Results

Except for 4 patients in groups 2 and 3, all others had RVNAT >0.5IU/ml (WHO recommended minimum protective level) on D0. All patients, showed high antibody titres on D14 following booster doses.

Discussion and recommendations

Four patients whose RVNAT was <0.5IU/ml on D0 had received only a partial course of ARV previously. As all subjects developed accelerated anamnestic antibody response on D14, recommendation of a full course of PET following a previous partial course of ARV within five years seems to be an over treatment. With these results, we could recommend two booster doses of ID ARV upto five years after even a partial course of ARV.
MRI research grant No.16-2007 is acknowledged.

OP 6

Comparison of recombinant protein and cell lysate antigens for detection of anti-chikungunya (CHIK) IgM antibody

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Introduction

Chikungunya (CHIK) virus specific antigen which has high specificity and low cross reactivity with other related diseases is required for laboratory confirmation.

Objective

To compare two antigens for detection of anti-CHIK antibody.

Design, setting and methods

In this study, two antigens (viral cell lysate and recombinant protein) were evaluated for detection of anti-CHIK antibody by using IgM ELISA. A novel recombinant protein antigen was designed based on envelope domain, a critical antigenic region of the major structural protein. This protein was expressed in *Escherichia coli* and resultant protein was affinity purified and ~10mg with >95% of purity per liter of culture was obtained. Cell lysate antigen was prepared using a crude culture fluid. Two antigens were evaluated separately using a panel of well characterized serum samples obtained from the Dept. of Virology (WHO Reference Centre for Viral Reference and Research), Institute of Tropical Medicine, Nagasaki University.

Results

A total of 64 serum samples confirmed as positives and 22 confirmed as negatives were used to evaluate the antigens. Specificity and sensitivity of the recombinant protein antigen was 48% and 90% respectively. Specificity and sensitivity of the viral lysate antigen was 17% and 100% respectively.

Viral lysate antigens can cause biohazard risk, high production cost and cross reactivity with other organisms of the same genus/family. Recombinant protein antigen which shows high specificity and sensitivity used in this study is important to overcome problems associated with viral lysate antigen. Testing of a large number of samples is needed to reconfirm this finding.

Acknowledgment

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OP 7

Detection of influenza A and B antigens in paediatric patients

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Introduction

Influenza A virus (H1N1) caused a worldwide pandemic from 2009 to 2011. Symptoms of this respiratory illness ranged from classical flu to severe pneumonia. In Sri Lanka, too, there were suspected and laboratory confirmed cases of influenza in the period, with varying severity. Of the clinically reported influenza cases, it is important to identify how many were positive for influenza A virus to make the initial link of the presenting illness to the suspected pathogen.

Objective

To detect influenza A/B antigens in clinically suspected flu patients.

Method

From June to Dec 2010, nasopharyngeal aspirates (NPA) and clinical details were collected from 70 children suspected of having severe flu (age = 2 months-15 years) from Teaching Hospitals Peradeniya and Kegalle and Sirimavo Bandaranayake Specialized Children's Hospital. Laboratory diagnosis was performed using QuickVue Influenza A+B chromatographic test kit (USA) which allows detection of influenza A/ B antigens in NPAs (Sensitivity = 95%; Specificity = 95%).

Of the 70 patients, 11 had cough, cold and fever; 5 had cough, cold, fever and wheezing and others had cough or cold without fever. Six patients had severe respiratory tract infection with 2 requiring ventilation. Of the 70 patients only 5 were positive for influenza A or B (7.14%). Although there was a suspicion, we were able to detect influenza A in only 3 patients. This shows the over suspicion of a pathogen because of panic and awareness during an epidemic. Most importantly, majority of the respiratory infections/diseases reported in the study might have been due to other infective causes which need further investigation.

Acknowledgement

We acknowledge Prof. J. S. M. Peiris, University of Hong Kong for providing guidance and QuickVue Influenza A+B test system (USA).

OP 8

Clinical and virological features of dengue in 2010

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Introduction

Dengue is an important viral infection in Sri Lanka. All 4 serotypes co-circulate in Sri Lanka.

Objective

To study the clinical and virological features of dengue in 2010.

Design, setting and methods

A hospital-based study was carried out at North Colombo Teaching Hospital, Ragama in 2010. Patients clinically suspected of having dengue, with fever less than 5 days were recruited. Acute and convalescent blood samples were collected within 7 days after obtaining informed written consent. Demographic, clinical information and laboratory results were obtained. Acute serum samples were tested using molecular (RT-PCR and Semi-Nested PCR) and serological (ELISAs and HAI) assays. Convalescent samples were tested by serological assays.

Results

Of 209 patients enrolled, 93 % (195/209) were laboratory confirmed as recent positive cases of dengue viral infection; of these, 5% (9/195) were classified as dengue fever; 85%(165/195) dengue haemorrhagic fever (DHF) and 0.5% (1/195) dengue shock syndrome. Mean platelet

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Introduction

Superficial fungal foot infection (SFFI) in diabetic patients increases the risk of developing diabetic foot syndrome. Sixteen percent of urban population is suffering from diabetes in Sri Lanka. Higher percentage (85%) of patients with long term diabetes is suffering from SFFI. As the diabetec patients are more prone to get fungal foot infections, early intervention is advisable owing to the progressive nature of the infection and to prevent from recurrent SFFIs which could even lead to diabetic foot syndrome. There is no documented data on the prevalence of fungal foot infections in diabetic patients in Sri Lanka.

Objective

To determine the etiological agents causing SFFI in patients with type 2 diabetes.

Methods

This descriptive cross sectional study included 385 out patients from the diabetic clinic at Colombo South Teaching Hospital. Nail clippings and swabs were collected from the infected sites using the standard protocol. Laboratory identification was done and pathogens were identified to the species level by direct microscopy, culture and biochemical tests. Statistical analysis was performed with the software SPSS version 15, using odds ratio and Chi-square tests.

Results

Clinically 295 patients (77%) showed SFFI, of which 255 (86%) were mycologically confirmed for nail infection and 46 (16%) were confirmed for both skin and nail infection. Out of 236 direct microscopy (KOH) positives, 227 (96%) were culture positive. Two hundred fifty one patients (98%) with SFFI had diabetes for more than 10 years. Of the patients with SFFIs 92% had >100 mg/dl FBS and 81% had >140mg/dl PPBS levels and 80% had both elevated FBS and PPBS levels. Non-dermatophyte fungal species were the commonest pathogens followed by Yeast and dermatophytes.

Conclusion

Aspergillus niger was the commonest cause of SFFIs in diabetes patients in the study setting followed by *Candida albicans*. Occurrence of SFFI was significant with the increasing age, gender, duration of diabetes and with less glycaemic control. Diabetic patients require regular foot examination and education on preventive care practices, as they are at high risk of developing SFFI.

OP 13

Development of recombinant protein antigens using a bacterial expression system for the detection of anti-Chikungunya (CHIK) antibodies

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Introduction and objective

Laboratory confirmation of Chikungunya (CHIK) virus is very useful as clinical symptoms of CHIK can overlap with other diseases. Chikungunya virus specific antigen, which shows high specificity, sensitivity and low cross reactivity with other related diseases, is required for laboratory confirmation. Objective of this study was to develop and compare two recombinant protein antigens for detection of anti-CHIK antibodies.

Design, setting and methods

Recombinant CHIK protein antigens were prepared using Envelope (E1 and E2) regions of the CHIK virus. The genes were custom designed and chemically synthesized with a 6X His tag. Bacterial expression systems [BL21 (DE3)] were used to clone and express the recombinant proteins. The recombinant proteins were purified with >95% of purity per liter of culture using Ni-NTA columns under denature conditions. In this study, two antigens were evaluated for detection of anti-CHIK antibody by using novel optimized in-house IgM and IgG ELISAs, using a panel of well characterized serum samples obtained from the Dept. of Virology (WHO Reference Center for Viral Reference and Research) Institute of Tropical Medicine, Nagasaki University, Japan.

Results

A total of 55 serum samples confirmed as positives and 186 confirmed as negatives by HAI test, IgM capture ELISA and indirect IgG ELISA using the purified CHIK antigen were used to evaluate the antigens using novel IgM ELISA. A total of 78 serum samples confirmed as positives and 148 (E1) or 227 (E2) (148 + extra 79) confirmed as negatives were used to evaluate the antigens using novel IgG ELISA. The E1 recombinant protein showed 5% (3/55) sensitivity and 99% (184/186) specificity for IgM ELISA and 60% (47/78) sensitivity and 63% (94/148) specificity for IgG ELISA. The E2 recombinant protein showed 65% (36/55) sensitivity and 70% (131/186) specificity for IgM ELISA and 83% (65/78) sensitivity and 86% (195/227) specificity for IgG ELISA.

Conclusion

Recombinant CHIK-E2 protein antigen showed higher specificity and sensitivity in detection of both IgM and IgG antibodies. Thus the E2 recombinant protein antigen used in this study could be expressed in an eukaryotic expression system to achieve much higher results.

Acknowledgment

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OP 14

Molecular evidence of hantavirus infection among clinically suspected patients with haemorrhagic fever with renal syndrome (HFRS)

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Introduction

Hantavirus disease is an emerging zoonotic viral infection with high fatality. Transmission is by inhalation of aerosols generated from virus contaminated rodent excreta. There are two major clinical forms, haemorraghic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). Clinical features of HFRS, often mimic leptospirosis. Large number of cases of leptospirosis like illness has been reported in Sri Lanka annually. Although t[illegible]e were serological evidence of different types of hantavirus infection in Sri Lanka, diagnosis of hantavirus is not routinely performed. Due to the genetic and antigenic diversity, an assay that could detect a wide range of hantaviruses need to be established.

Objectives

To establish, evaluate and validate a genus specific hantavirus RT-PCR assay.

To diagnose hantavirus infection among clinically suspected HFRS patients in three selected hospitals.To describe clinical manifestations of hantavirus infections in the study population.

Methodology

Genus specific conventional RT-PCR assay was established using panhanta primers and evaluated, optimized and validated using synthetic genes of 12 known hantavirus species as reference samples. Assay was able to detect a wide range of hantaviruses at minimum detection limit of 70 copies/ reaction.

Molecular diagnosis of hantavirus infection was carried out in three hospitals in Colombo and Gampaha districts. Study was conducted from 01st of January 2011 to 31st of April 2011 and 61 adult patients were recruited to this study.

Hantavirus RT-PCR was performed on all collected samples after extraction of RNA by TRIzol® method.

Results

Of 61 tested samples, 05 were positive for hantavirus genome. These results were confirmed at reference laboratory as well and species identification result is pending.

Of 58 tested samples, 06 samples were positive for hantavirus IgM by in-house ELISA. All PCR positive samples were positive for hanta virus IgM.

All patients with hantavirus infection had clinical and biochemical features of liver involvement in addition to fever, thrombocytopenia and renal involvement.

Conclusion

Established RT-PCR assay was able to detect a wide range of hantaviruses and by using it molecular evidence of hantavirus infection was demonstrated in humans in Sri Lanka.

Further studies are required to describe the disease epidemiology and to identify natural hosts in the country.

OP 15

Enhancing the sensitivity of methicillin-resistant *Staphyloccous aureus* (MRSA) to oxacillin by tea catechins and proanthocyanidins

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Introduction

The emergence of MRSA has become a disturbing clinical problem. New antimicrobials and novel strategies for treatment are urgently required to combat drug-resistance. The tea plant (*Camellia sinensis L.*) is a potential source of non-toxic and low-cost antimicrobials.

THE SRILANKA COLLEGE OF MICROBIOLOGISTS

22nd Annual Scientific Sessions

24th-26th July 2013

This is to certify that

Athapaththu AMMH, Khanna N, Inouye S, Gunasena S, Abeyewickreme W, Hapugoda M

is awarded the Second Prize *in Oral Presentations*

for the paper titled

Development of recombinant protein antigens using a bacterial expression system for the detection of anti-chikungunya (IgM) antibodies.

at the 22nd Annual Scientific Sessions 2013 held at Medical Research Institute, Colombo

S. Gunasena

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Athapaththu, A.M.M.H.
Khanna, N.
Inouve, S.
Gunasena, S.
Abeywickreme, W.
Hapugoda, M.
PAPER

Comparison of recombinant Chikungunya (CHIK) E2 antigens expressed in bacterial and eukaryotic systems for the detection of anti-CHIK antibodies in human serum samples

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Introduction :
Laboratory confirmation of Chikungunya Virus (CHIKV) is very useful as it shows the same symptoms as dengue and can be present in the same regions, at the same period. Currently, recombinant protein antigens are widely used as diagnostic intermediates due to biosafety, low production cost and minimum cross reactivity with other organisms of the same genus/family.

Objective:
To develop and compare the efficacy of recombinant protein antigens expressed in bacterial and eukaryotic systems.

Design, setting and methods:
The Envelop 2 (E2) protein in the E region of the CHIKV was selected for the preparation of recombinant protein as it is the more immunodominant region of the virus. The chemically synthesized E2 gene was cloned and expressed separately in *Eschericia coli* BL21 (DE3) and *Pichia pastoris* (KM71H). Each recombinant antigen was purified using Immobilized Metal-Affinity Chromatography (IMAC). Novel in-house Enzyme Linked Immunosorbent Assays (ELISAs) with recombinant protein antigens to detect anti-CHIK Immunoglobulin (Ig) M and G antibodies were developed. Field evaluation of novel ELISAs were performed using two panels of well characterized serum samples. A total of 55 serum samples confirmed as positives and 186 confirmed as negatives were used to evaluate the antigens using novel IgM ELISA. A total of 78 serum samples confirmed as positives and 227 confirmed as negatives were used to evaluate the antigens using novel IgG ELISA.
Sensitivity and specificity were calculated in each recombinant protein according to the following formula:
Sensitivity = Number of true positive
Number of true positive + false negative

Specificity = Number of true negative
Number of true negative + false positive

Results:

E. coli E2 recombinant protein showed 65% (36/55) sensitivity and 70% (131/186) specificity and *P. pastoris* E2 recombinant protein showed 49% (27/55) sensitivity and 78% (146/186) specificity in IgM ELISA. *E. coli* E2 recombinant protein showed 83% (65/78) sensitivity and 86% (195/227) specificity, *P. pastoris* E2 recombinant protein showed 76% (59/78) sensitivity and 81% (183/227) specificity in IgG ELISA.

Discussion:

E. coli E2 has showed better sensitivity and specificity in identification of both IgM and IgG antibodies. This could be due to more exposure of immunodominant epitopes of bacterial expressed E2 antigen to the outside.

110/A

Development of recombinant protein antigens using a yeast expression system, for the detection of anti-Chikungunya (CHIK) antibodies in clinical samples

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Laboratory confirmation of Chikungunya (CHIK) virus is very useful as clinical symptoms of CHIK overlap with those of other diseases. Laboratory diagnosis depends on Enzyme-Linked Immunosorbent Assay (ELISA) based on whole viral antigens which cause a biohazard risk, have a high production cost and show cross reactivity with other organisms of the same genus / family. A diagnostic intermediate using a single recombinant protein antigen to detect both immunoglobulin (Ig) M (IgM) and IgG antibodies of CHIK is important to overcome problems associated with whole viral antigen / lysate. The objective of this study was to prepare recombinant protein antigens for detection of anti-CHIK antibodies. Recombinant CHIK protein antigens were prepared using two Envelope (E) gene regions of the CHIK virus which were custom designed and chemically synthesized with 6X His tag. Yeast expression systems; *Pichia pastoris* (*P.pastoris*) were used to clone and express the recombinant proteins. Purification of proteins was performed using Ni-NTA columns under denature conditions. Novel in-house IgM and IgG ELISAs were developed using recombinant protein antigens. The two antigens were evaluated for detection of both IgM and IgG CHIK antibodies using a panel of well characterized serum samples. A total of 55 serum samples confirmed as positives and 186 confirmed as negatives were used to evaluate the antigens using novel IgM ELISA. A total of 78 serum samples confirmed as positives and 227 (148 and 79) confirmed as negatives were used to evaluate the antigens using novel IgG ELISA. These samples were tested by HAI test, IgM capture ELISA and indirect IgG ELISA using the purified CHIK antigen and Infected Culture Fluid (ICF). The E1 recombinant protein showed 15% (8/55) sensitivity and 97% (181/186) specificity for IgM ELISA and 86% (67/78) sensitivity and 61% (90/ 148) specificity for IgG ELISA. The E2 recombinant protein showed 49% (27/55) sensitivity and 78% (146/186) specificity for IgM ELISA and 76% (59/78) sensitivity and 81% (183/227) specificity for IgG ELISA. Recombinant protein antigens expressed in yeast showed lower specificity and sensitivity than expected for detection of both IgM and IgG anti-CHIK antibodies, but E2 recombinant proteins performed better than E1recombinant antigen as it is exposed more to the human immune system than E1 protein.

Keywords: Chikungunya, recombinant protein antigens, E1, E2, ELISA

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PAPER

Detection of pathogenic *Leptospira* species in rat blood samples by molecular-based assays[12]

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Background:
Leptospirosis is a worldwide zoonotic infection, caused by pathogenic species of the genus *Leptospira*. It was traditionally known as 'rat fever' in Sri Lanka, because rodents, especially rats, are considered to be the most important reservoirs or maintenance hosts of *Leptospira*. In 2012, the highest numbers of cases were reported in the District of Gampaha. The objective of this study is to detect pathogenic *Leptospira* species in rat blood samples by molecular based assays.

Method:
Rats (n=38) were trapped in a high risk area (Mirigama) in the District of Gampaha, from May 2012 to February 2013 by using live traps. Each rat was anesthetized by using diethyl ether and 2-3 ml sample of blood was collected from each rat. Blood samples collected from all rats were tested by molecular- based assays and a serological assay. Qualitative Polymerase Chain Reaction (PCR), real time PCR and Loop Mediated Isothermal Amplification (LAMP) were used as molecular-based assays which targetted conserved gene regions among pathogenic serovars of *Leptospira* species. Microscopic Agglutination Test (MAT), the Gold Standard assay for detection of anti *Leptospira* antibody was used as a serological assay.

Results and Discussion:
Of the 38 rat blood samples, molecular-based assays confirmed *Leptospira* infection in 5% (2/38), 16% (6/38) and 11% (4/38) by qualitative PCR, real time PCR and LAMP assay respectively. None of the samples was positive by MAT. After first infection, some *Leptospira* species live in the host animal as commensal bacteria. Therefore, host does not stimulate antibody production further and that may be below the detection level of the antibody by MAT.

Conclusions:
Results of molecular based assays showed that *Leptospira* are circulating among the rats tested in this study, although at the time of collection, their antibody levels were too low to detect by MAT, which had the lowest detection limit of 1:800.

[12] **Acknowledgements:** National Science Foundation, Sri Lanka (NSF/RG/2009/BT/01), International Centre for Genetic Engineering and Biotechnology (ICGEB CRP/ SRI 08-02) and International Atomic Energy Agency (IAEA SRI 5/042) are gratefully acknowledged.

Denipitiya, D.T.H.
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PAPER

Risk factors associated with human leptospirosis in the District of Gampaha, Sri Lanka[13]

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Background & Objective:
A large number of leptospirosis cases are recorded in Sri Lanka every year. Increased numbers of cases have been reported in the District of Gampaha in the recent past. The incidence of leptospirosis is often influenced by various socio-economic, occupational, environmental and other factors. To date, a study on potential risk factors has not been conducted in the District of Gampaha. The objective of this study is to identify risk factors involved in transmission of leptospirosis to humans in the District of Gampaha.

Methods:
Data were collected at the household level, using an interviewer-administered questionnaire and by inspecting the surrounding of laboratory confirmed leptospirosis patients (n=81) and non leptospirosis persons (n=117) during the period of June 2011 to June 2013. The risk factors in the questionnaire were divided into three broad categories: environmental, contact with animals and behavioral/occupational factors. Chi-square test (The SAS System for Windows 9.0) was used for comparison of data from different categories.

Results and discussion:
95% of the leptospirosis patients were adult males (77/81) and they had a monthly income of Rs. 10,001-20,000 and 50% of them were agricultural and rental work labourers (40/81). In contrast, 56% of persons not infected with leptospirosis were adult females (66/117) and most of them (48%) were housewives or homemakers (56/117). Data on the type of premises were collected under three categories as poor, moderate and well constructed along with the land use type of the surrounding areas. There were significant statistical associations between the leptospirosis patient with the type of premises (, χ^2=23.38, p=0.00), surrounding cleanliness of premises (χ^2=45.05, p=0.00), sanitary facilities (χ2=11.66, p=0.00), waste disposal method (χ2=32.23,

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Evaluation of a real time Polymerase Chain Reaction (PCR) assay for the early diagnosis of human leptospirosis

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Leptospirosis has the greatest impact on health in developing countries where it is often grossly under-recognized due to difficulties in clinical diagnosis and lack of diagnostic laboratory services. The disease is usually diagnosed in the laboratory by serological techniques or by culturing of bacteria from clinical specimens which results in delayed diagnosis. The objective of this study was to establish and evaluate a real time Polymerase Chain Reaction (PCR) assay using SYBR green for early, rapid and definitive laboratory diagnosis of leptospirosis. The assay was established and analytical specificity and sensitivity were determined using reference DNA samples. The accuracy of the real time PCR assay was determined using a panel of acute blood samples collected from leptospirosis (n = 60) and non leptospirosis (n = 51) confirmed patients based on serological assays. The analytical sensitivity of the assay was approximately 58.8 genome equivalents per reaction and no cross-reactivity was observed with saprophytic *Leptospira* spp. and other pathogenic micro organisms. The assay successfully detected leptospiral DNA from blood samples of clinically diagnosed patients with leptospirosis and showed high diagnostic sensitivity 82.05% (32/39) and specificity 80.55% (58/72). This study showed that real time PCR has the potential to facilitate rapid and sensitive diagnosis of acute leptospirosis during the early phase of infection.

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Evaluation of a case definition for leptospirosis diagnosis using serological assays

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Leptospirosis has been a public health problem in many parts of Sri Lanka and has had a great effect on the economic and social development of the country. A large number of leptospirosis cases are recorded in Sri Lanka every year. Leptospirosis is often mistaken for other acute febrile illnesses because of its nonspecific presentation. All bacteriologic, serologic, and molecular methods are expensive and available only at reference laboratories, especially in developing countries like Sri Lanka. For proper management of leptospirosis patients, it is important to diagnose leptospirosis as soon as possible after admission to a hospital. This study was aimed to evaluate the leptospirosis surveillance case definition using serological assays. Adult patients, admitted to the medical wards of the two main hospitals of the District of Gampaha from 1st of January 2011 to 31st of December of 2012 with undiagnosed acute febrile illness were included to test a case definition of the WHO guidelines-2003. Serological assays were performed on blood samples taken from each patient after 7 days of fever. Leptospirosis case definition was evaluated with regard to sensitivity, specificity and predictive values using a MAT titer ≥ 1:800 and/or positive band in commercially available IgM kit for confirming leptospirosis. Of 191 patients, the clinical features of 107 only were compatible with the surveillance case definition. Of the 107 only 60 were true positives and 47 were false positives based on serological assays. Out of the 84 only 4 were false negatives. According to these results the test sensitivity was 93.75% (60/64) and specificity was 62.99% (80/127). Positive predictive values and negative predictive values were 56.07% (60/107) and 95.27% (80/84) respectively. This study confirms that the surveillance case definition has a very high sensitivity and negative predictive value with an average specificity in diagnosing leptospirosis based on serological assays.

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A COMPARISON OF THREE MOLECULAR-BASED ASSAYS TO DETECT PATHOGENIC LEPTOSPIRES IN CATTLE URINE

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Leptospirosis is a globally widespread, emerging zoonotic disease that poses important public health threats in humid, tropical and subtropical areas. Leptospirosis is transmitted directly or indirectly from animals to humans through contact with contaminated soil, water, or body fluids of infected animals. Most feral and domestic mammals may serve as major reservoir hosts for *Leptospira* spp. However, there is little knowledge on the role of wild mammals, including the large number of rodents, as reservoir hosts of leptospires in Sri Lanka. The objective of this study was to compare three molecular-based assays to detect pathogenic leptospires in cattle urine samples. Mid-stream urine samples were collected from 50 cattle in a high risk area for leptospirosis (Mirigama) in the Gampaha district from May 2012 to February 2013. A Loop-Mediated Isothermal Amplification (LAMP) assay for the rapid detection of pathogenic *Leptospira* species was established using reference samples (*L. interrogans* strain RGA) through amplification of the lipL41 gene coding for the outer membrane protein LipL41. Qualitative Polymerase Chain Reaction (PCR) and quantitative real time PCR assays were established targeting a 202 bp fragment on the secY gene which is conserved among pathogenic serovars of *Leptospira*. Analytical sensitivity and specificity of each assay were tested using reference DNA samples. Each urine sample was tested by three molecular-based assays; namely, LAMP, qualitative PCR and quantitative real time PCR, with positive and negative controls. The repeatability of each assay was tested using two replicates of each sample. Concentrated urine samples were mixed with reaction buffer and directly applied for LAMP reaction. DNA was extracted from concentrated urine samples using a commercially available QIAGEN kit and the extracted DNA was used for both PCRs. Each molecular assay showed 100% analytical specificity. The analytical sensitivity (per reaction) of LAMP assay, qualitative PCR, and real time PCR were 5.8, 588, and 58.8 copies of bacteria, respectively. Of the 50 cattle urine samples, molecular-based assays confirmed *Leptospira* infection in 70% (35/50), 2% (1/50), and 10% (5/50) by LAMP assay, qualitative PCR, and real time PCR, respectively. In conclusion, we established three molecular-based assays to detect pathogenic *Leptospira* species in cattle urine samples. The highest number of positive reactors was detected with the new LAMP assay which utilises a simple DNA preparation step to detect pathogenic *Leptospira* species in urine. In contrast to PCR assays that use purified DNA samples from urine, the LAMP assay can amplify the target DNA without DNA purification and boiled urine samples are sufficient to prepare the DNA template. The results of these molecular-based assays showed that *Leptospira* spp. are circulating among the cattle tested in this study and pose a public health threat to farmers and farm workers in this area.

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