

1. Product Development

Dr. K. Balaraman, DD (SG)	Dr. M. Kalyanasundaram, DD (SG)
Dr. S.L. Hoti, DD	Dr. A.M. Manonmani, AD
Dr. N. Pradeepkumar, AD	Dr. S. Poopathi, AD
Dr. K.P. Paily, SRO	Dr. Nisha Mathew, SRO
Dr. V. Vasuki, TO	Dr. K.P. Patra, RA
Mrs. Abida, RA	Mrs. K. Athisaya Mary, RA
Mr. G. Prabakaran, RA	Mrs. I. Geetha, RA
Mrs. J. Ramyavani, RA	Mr. V. Padmanabhan

1.1. Identification of epitope(s) binding *Wuchereria bancrofti*-specific monoclonal antibody, VCRC B5 (V.Vasuki & S. L. Hoti; IM 0406 PD; Duration: 3 years, Oct 2004 – Sep 2007)

Monitoring exposure of human population in an endemic community to *W. bancrofti* infection, especially during the later phase of a lymphatic filariasis (LF) elimination programme, is important. However, the tools necessary for monitoring exposure are not available. Detection of filarial-specific antibody will provide information on the exposure of the community to infection. Currently available commercial immuno-diagnostic kits for filariasis, such as Og₄C₃ Enzyme Linked Immuno Sorbant Assay (ELISA) and Immuno Chromatographic Test (ICT) card, detect circulating filarial-specific antigens in the blood, but not antibodies. In an earlier study a monoclonal antibody (VCRC B5), specific for L3 stage larva of *W. bancrofti*, which has high specificity and sensitivity (90-100%), was identified. The present study has been initiated to identify the antigenic epitope(s) recognized by this monoclonal antibody and to characterize the gene coding for the corresponding antigenic epitope(s). The crude monoclonal antibody (VCRC B5) has been purified to the level of 99% purity by affinity column chromatography and assessed for its antigenic reactivity with filarial antigens. It is currently being used for screening the L3 stage cDNA library of *W. bancrofti*.

1.2. Evaluation of infective (L3) stage-specific RT-PCR assay for the detection of *Wuchereria*

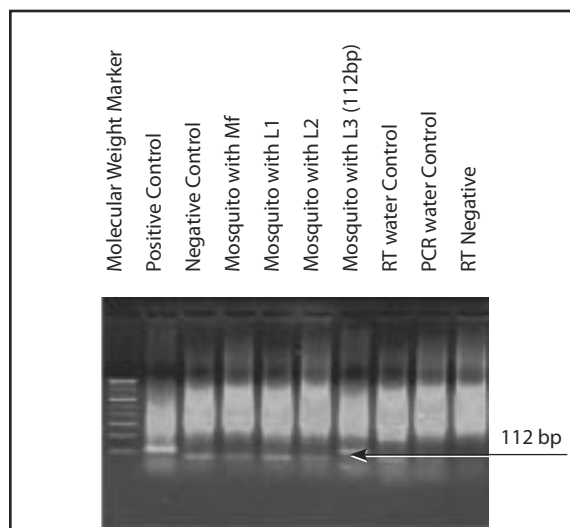
bancrofti L3 in vectors (V.Vasuki & S.L.Hoti; IM 0203 PD; Duration: 2 years, Nov 2002 – Dec 2004)

Xenomonitoring is important for particularly assessing the risk of transmission of *W. bancrofti* infection in an endemic area. This can be done by detecting the presence of infective (L3) stage larvae in vectors and is being done by the conventional method of dissection and microscopic examination. However, this method is cumbersome and hence not suitable for large-scale xenomonitoring. Molecular biological tools such as polymerase chain reaction (PCR) assays offer high sensitivity and specificity, rapidity and amenability for mass screening. PCR assays have been developed to determine vector infection rates of *Wuchereria bancrofti*, *Brugia malayi* and *Onchocerca volvulus*. However, these assays can detect any stage of the filarial species in vectors, but not L3 stage of the parasite. Therefore, stage-specific probes for the detection of L3 stage larvae were earlier developed at the VCRC and evaluated for the detection of L3 larva in the vector mosquito, *Culex quinquefasciatus*, during 2004.

The validation of the infective (L3) stage-specific probe was undertaken in two phases. Phase I aimed at assessing the stage specificity of the probe, wherein samples of mosquitoes having either microfilaria (Mf), L1, L2 or L3 stage of the parasite and uninfected mosquitoes were assayed by Reverse Transcriptase-PCR (RT-PCR). The probe could detect mosquitoes having a minimum number (1-2) of L3 and was able to detect only L3 among all other stages, with an amplicon of 112 bp (Fig 1.1). Phase II evaluation is

going on to determine the sensitivity of the probe in samples of infected and uninfected mosquitoes.

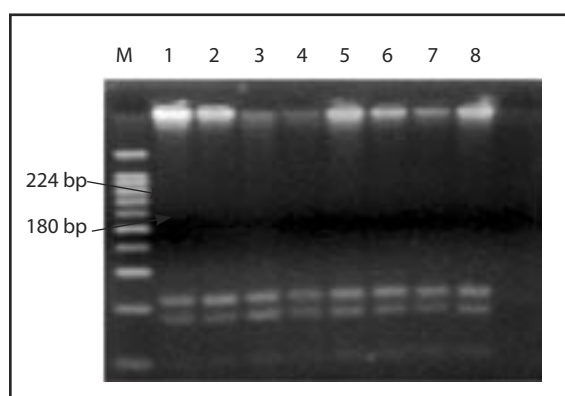
Fig. 1.1. Stage specificity of RT-PCR assay for detecting infective stage (L3) larvae of *W. bancrofti* in mosquitoes



1.3. Role of VEGFR-3 and FOXC2 in primary lymphoedema as risk factors in the pathogenesis of LF (S.L.Hoti, P.K.Das, & L.K. Das; IM 0407 PD; Duration: 5 years, Oct 2004 – Sep 2009)

Recent studies have shown that mutations in VEGFR-3, (G3123C SNP) and FOXC2 (C939A SNP) genes are involved in primary and secondary lymphoedema, respectively, in humans. External agents trigger the pathogenesis. This study was to investigate the role of mutations in VEGFR-3 and FOXC2 genes in lymphatic filarial pathogenesis. Techniques to genotype

Fig. 1.2. Genotyping of filarial lymphoedema patients for VEGFR-3 alleles (3123 SNP). (M – 100 bp ladder marker, 1 to 8 – patient samples)



VEGFR-3 alleles have been standardized. So far, 21 blood samples from microfilaraemic individuals and filarial lymphoedema patients, residing in Pondicherry, have been collected and are being used for genotyping of VEGFR-3 gene of patients. Of these, eight blood samples were screened for VEGFR-3 and all of them were found to be heterozygous genotypes, yielding bands for normal (224 bp) and mutant (180 bp) allele (Fig.1.2). One of the patients, who had congenital lymphoedema, also showed the heterozygous genotypic character for VEGFR-3 allele.

1.4. Role of endosymbiont, Wolbachia sp., in the pathogenesis of LF (S.L.Hoti, P.K. Das, K.A. Mary & L.K. Das; IM 0408 PD; Duration: 5 years, Oct 2004 – Sep 2009)

Rickettsia-like endosymbionts, *Wolbachia* sp., of filarial nematodes have been implicated in morbidity due to filariasis, apart from their role in elicitation of adverse reactions following antifilarial drug (DEC) treatment. This project was therefore undertaken (i) to investigate the role of Lipopolysaccharide (LPS), *Wolbachia* Surface Protein (WSP) and other bacterial antigens and/or parasite antigens; and (ii) to assess the severity of adverse reactions vis-à-vis the release of endosymbiont or its molecules and the inflammatory reaction following DEC treatment. Techniques to detect *Wolbachia* endosymbiont by PCR (16s RNA, *ftz* and *wsp* genes) have been standardized.

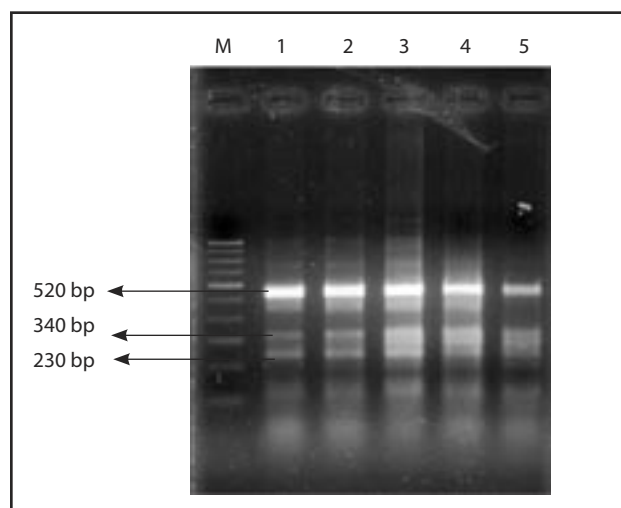
1.5. Role of HLA and non-classical HLA genes in dengue clinical spectrum in Pondicherry and identification of epitopes (S.L. Hoti & K. Balaraman; IM 0408 PD; Duration: 3 years, Oct 2004 – Sep 2007)

Dengue Hemorrhagic Fever (DHF), the more severe form of dengue infection, is defined by fever, hemorrhage, thrombocytopenia and plasma leakage. The exact patho-physiological mechanisms behind this phenomenon are not fully understood. Earlier studies in Thailand, Mexico and Cuba found that certain Class I alleles of the

Human Leukocyte Antigen (HLA) complex are associated with resistance or susceptibility to DHF. Transporter Associated With Antigen Processing (TAP), a non-classical HLA protein, functions as a channel for transport of processed peptides into the endoplasmic reticulum (EPR) for loading onto Major Histocompatibility Complex (MHC) Class I molecule. This channel allows only a few of the viral peptides to pass through for loading onto MHC molecule although many of them are produced. Thus, TAP, in association with MHC, plays a key role in dengue infection and their genotypes may have a role in the clinical spectrum. Therefore, the present project was taken up with the following objectives: (i) to assess the role of HLA Class I and II, and non-classical HLA gene TAP allele polymorphism in clinical outcome of dengue viral infection; and (ii) to demonstrate the immunogenic potential of dengue-specific peptides against the HLA allele with high frequency among Pondicherry people, and their validation with patient sera.

Amplification of TAP-1 gene from DNA of dengue patients has been standardized (Fig. 1.3). Eleven blood samples from confirmed dengue cases and normal individuals residing in Pondicherry have been collected and are being processed for genotyping. Four samples were subjected to genotyping and of them one case of DHF was homozygous for TAP-1 allele, while three normal controls were heterozygous. In order to understand the immunogenic potential of different peptides of the virus, complete amino acid sequences of all 10 proteins of dengue virus cell envelope were deduced and were analyzed for their ability to bind to HLA Class I alleles as well as Mouse MHC Class I alleles *in-silico*. The algorithm generated all possible overlapping nonameric peptides and identified those peptides that contain binding motifs for MHC Class I alleles. The binding is measured in terms of half time of dissociation rate of $\beta 2$ microglobulin, which is designated as $T_{1/2}$ of dissociation. A few peptides of dengue virus, which showed binding to any of the 33 MHC alleles with $T_{1/2} > 100$, were identified and are being taken up for further analysis (Fig. 1.4).

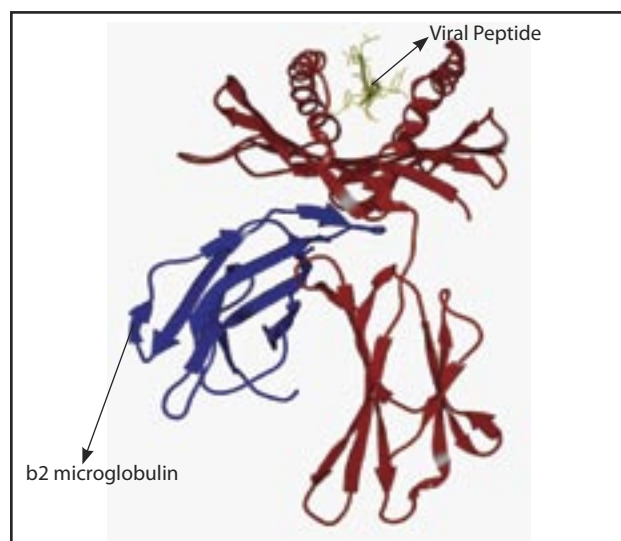
Fig.1.3. Optimization of genotyping for TAP-1 allele from a normal human. (M – 100 bp ladder marker, 1 to 5 - $MgCl_2$ concentrations)



1.6. Synthesis of 1-Methyl-4-substituted benzoyl piperazides for the development of a macrofilaricide (M. Kalyanasundaram, Nisha Mathew, K.P. Paily & G. Prabakaran; EM 9909 PD; Duration: 3 years, Jan 2003 – Dec 2005)

Currently available drugs for the control of LF are only microfilaricidal. Therefore, development of a macrofilaricide based on the inhibition of possible biochemical targets is given much importance. The present study aims at synthesizing 1-Methyl-4-substituted benzoyl piperazides, screening the compounds

Fig. 1.4. Binding of dengue viral peptide to the MHC Class I structure



for their efficacy under *in vitro* conditions against *Setaria digitata* adults, and inhibition of the target enzymes, 5,10-methylenetetrahydrofolate reductase and serine transhydroxymethyltransferase.

During the year 2004, eleven compounds (DSTD-10 to DSTD-20) were synthesized and the purity of the compounds was tested by thin layer chromatography, using 5% methanol in chloroform as the eluting phase on a pre-coated silica gel plate. The R_f factors of the compounds from DSTD-10 to DSTD-20 are 0.67, 0.60, 0.44, 0.39, 0.33, 0.29, 0.31, 0.37, 0.36, 0.44 and 0.69 respectively. The compounds were characterized by infrared spectra, which showed a strong absorption around 1625 – 1640 cm^{-1} characteristic for amide carbonyl.

Out of 20 substituted 1-Methyl-4-substituted benzoyl piperazides synthesized so far and tested, six compounds, namely, DSTD-1, 11, 12, 13, 14 & 15 were found to cause 100% inhibition in the mobility of *Setaria digitata* adult worms at 10 mg/ml and were tested at lower concentrations. The results showed that DSTD-1, 11, 12, 13, 14 & 15 were effective at 3, 7, 10, 10, 9 & 8 mg/ml. The structure-activity relationship indicates that substituted piperazides with chlorine in 2, 3 and 4 positions of the phenyl ring of the phenyl-acetic acid exhibited activity and those with methoxy and methyl groups were not effective. These compounds need to be studied for enzyme inhibition against the targeted enzymes and the method is currently being standardized.

1.7. Synthesis of structural analogues of the macrofilaricidal lead molecules, P-19 and P-21 and screening (Nisha Mathew, M. Kalyanasundaram & K. Balaraman; IM 0302 PD; Duration: 5 years, Nov 2003 – Oct 2008)

From earlier work two lead molecules exhibiting macrofilaricidal activity were identified from medicinal plants. Seven analogues of the lead molecule viz., 3-substituted amino-5-hydroxy-2-methyl-1,4-naphthoquinones have been

synthesized and screened for macrofilaricidal activity against *Setaria digitata* by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay (VCRC Annual Report, 2003).

On 16th March, 2004 an MOU was signed between VCRC, Pondicherry and CDRI, Lucknow to test the lead molecules in animal models for macrofilaricidal activity. Structure-activity relationship studies showed that the macrofilaricidal potential of 5-hydroxy-2-methyl-1,4-naphthoquinone is influenced by the substitution at C3 carbon of the dione ring; the substitutions by 3-dimethyl amino-1-propyl amino, 3-diethyl amino-1-propyl amino and 3-butylamino-1-propyl amino groups reduced the macrofilaricidal activity while substitutions with dimethylamino, butylamino, propylamino and isopropylamino groups enhanced macrofilaricidal activity. Now seven more compounds have been synthesized and these compounds have to be screened for macrofilaricidal activity by MTT reduction assay.

1.8. Suppression of *Wuchereria bancrofti* development in *Culex quinquefasciatus* exposed to biolarvicides (K.P. Paily, S.L. Hoti & K. Balaraman; EM 9908 PD; Duration: 3 years, Jul 2002 – Jun 2005)

The objectives of the project are (i) to study the effect of residual bacterial toxin on the infection and development of *W. bancrofti* in treated mosquito; (ii) to study the effect of changes in the mosquito gut lectins on the parasite infection; and (iii) to study the effect of mosquito defense proteins acquired due to exposure to bacterial larvicides, on parasite development.

Biolarvicides such as *Bacillus sphaericus* (*B.s*) and *Bacillus thuringiensis* (*B.t*) are used for the control of mosquitoes. Apart from their known effects on vector density, there are reports of reduced vector potential when the larvae are exposed to sub-lethal doses of biolarvicides. However, the exact mechanism of suppression

of parasite development in mosquitoes exposed to biolarvicides is not known. Therefore, a study was undertaken to investigate this. Mosquito larvae exposed to *B.s* and *B.t* exhibited transtadial transmission of the bacteria (VCRC Annual Report, 2003). Hence, there was a possibility of its having an adverse effect on the development of *W. bancrofti*.

When the mosquito stages of *W. bancrofti* viz., Mf, L1, L2 and L3, were exposed to the digested toxins of *B.s* and *B.t*, it was observed that their motility was not affected. Larvae of *C. quinquefasciatus*, which were exposed to a sub-lethal dose of the biolarvicides, and pupae and adults which survived the treatment, were examined for changes, if any, in the lectin level. Remarkable increase in the level of lectin was noticed in adults which emerged from *B.t* treated larvae, whereas no significant changes have taken place in the larvae and pupae; and the lectin was not GlcNAc-specific (Table 1.1). In contrast to this, the level of lectin was not increased in the adults that emerged from *B.s* treated larvae and, interestingly, there was an appreciable increase in the larvae and pupae. However, the lectin was specific to GlcNAc.

These results suggest that in *B.t* exposed mosquitoes the level of GlcNAc- non-specific lectin is enhanced, which may have a role in suppressing the development of mosquito

stages of *W. bancrofti*. Apart from confirming the role of GlcNAc- non-specific lectin in the suppression of *W. bancrofti* development, other factor(s) that may play a similar role may have to be investigated. In *B.s* exposed mosquitoes there was no such increase in GlcNAc- non-specific lectin level. Therefore, the suppression of mosquito stages of *W. bancrofti* is governed by factor(s) other than GlcNAc- non-specific lectin. This is being investigated.

1.9. Mosquito humoral factors involved in the regulation of *Wuchereria bancrofti* development in *Culex quinquefasciatus* (K.P. Paily & K. Balaraman; EM 0104 PD; Duration: 3 years, Jan 2003 – Dec 2005)

The objectives of the project are (i) to identify and characterize the mosquito humoral factors involved in the defense against facilitation of *W. bancrofti* infection and development in *Cx. quinquefasciatus*, and (ii) to identify and characterize the vector transcripts corresponding to the filarial-specific haemolymph proteins produced during infection and development of *W. bancrofti*.

Mosquitoes are known to possess immune factors against invading parasites. However, no information is available regarding such factors against *W. bancrofti* in *C. quinquefasciatus*, the vector of bancroftian filariasis. Identification of

Table 1.1. Results of hemagglutination and GlcNAc inhibition assay for lectins in *B. sphaericus/B. thuringiensis* treated *Cx. quinquefasciatus*

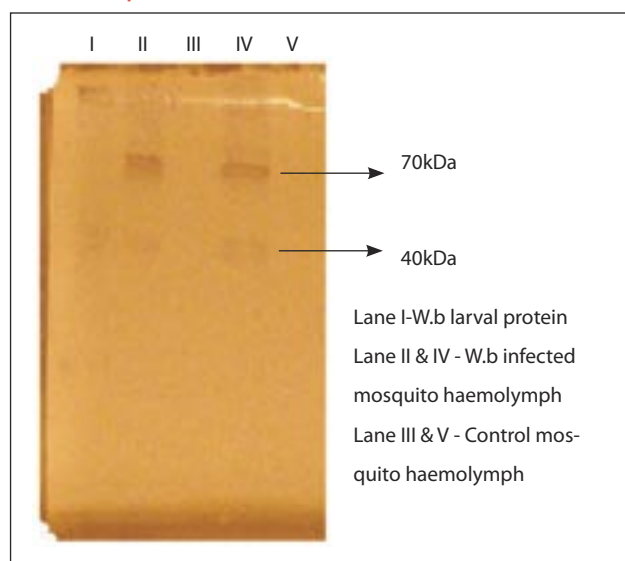
Sample	Agglutination titer	Protein (µg/ml)	Inhibition titer	Protein (µg/ml)
Control larva	1 : 8	0.00625	1 : 4	0.01250
Control pupa	1 : 8	0.00625	1 : 4	0.01250
Control adult	1 : 8	0.00625	1 : 2	0.02500
<i>B. t.</i> treated larva	1 : 8	0.00625	1 : 4	0.01250
<i>B. t.</i> treated pupa	1 : 2	0.02500	1 : 2	0.02500
<i>B. t.</i> treated adult	1 : 64	0.00078	1 : 64	0.00078
<i>B. s.</i> treated larva	1 : 64	0.00078	1 : 32	0.00156
<i>B. s.</i> treated pupa	1 : 32	0.00156	1 : 32	0.00156
<i>B. s.</i> treated adult	1 : 8	0.00625	1 : 2	0.02500

the humoral immune factors in *C. quinquefasciatus* might lead to the development of susceptibility markers as well as transmission monitoring tools. In our study, haemolymph of *W. bancrofti* infected *C. quinquefasciatus* was found to have three new proteins and three over-expressed proteins (VCRC Annual Report, 2003).

Out of six proteins, five (mw: 70, 66, 40, 22, and 14 kDa) were purified, antisera developed and tested by immunoblotting against the homogenate of *W. bancrofti* larvae as well as the haemolymph of uninfected and *W. bancrofti* infected mosquitoes. The antisera reacted with 70 and 40 kDa proteins present in the haemolymph of infected mosquitoes (Fig. 1.5). The antisera did not react with the homogenate of *W. bancrofti* larvae or haemolymph proteins of uninfected mosquitoes.

The results indicate that the 70 and 40 kDa proteins are of mosquito origin and synthesized *de novo* following infection by *W. bancrofti*. These proteins, being markers of *W. bancrofti* infection in *C. quinquefasciatus*, need to be characterized. The other three proteins, viz., 66, 22 & 14 kDa, although eliciting a high titre of antibody production, did not react with the haemolymph of *W. bancrofti* infected mosquitoes, in spite of the tests being repeated four times. This needs to be investigated.

Fig. 1.5. Immunoblot of mosquito haemolymph proteins



1.10. Characterization of the mosquitocidal strains of *Bacillus subtilis* group, VCRC B469, B471, B474 and their metabolites (K. Balaraman, A.M. Manonmani, K.P. Paily, G. Prabakaran & I. Geetha; IM 0401 PD; Duration: 2 years, Jun 2004 – May 2006)

This project was taken up to develop three potential strains of *B. subtilis* group for mosquito control through molecular, biochemical and toxicological studies. In our attempts to develop potential microbial agents for mosquito control, 12 bacteria were found to possess larvicidal/pupicidal properties. Out of these, three isolates belonging to *B. subtilis* group, viz., VCRC B469, VCRC B471 & VCRC B474, were more potent (Annual Report, 2003). During 2004, studies were carried out to determine their identity at molecular level, characterize the metabolites, and test their safety to mammals.

Identity: The results of molecular identification indicated that VCRC B469 and VCRC B471 belong to *B. subtilis* 16s RNA group (Fig. 1.6). Their PCR products are being sequenced for confirmation of the identity. As for identification of the strain, VCRC B474, experiments are underway.

Mammalian safety: Acute oral toxicity/pathogenicity tests conducted on Wistar rats, with formulations of the three strains of *B. subtilis* group at about 50 times the LC_{50} dosage, showed that they are non-toxic because there were no adverse effects on body weight, food consumption, rectal temperature, haematological and biochemical parameters. In the primary skin irritation test conducted on rabbits at a similar dosage, the index of each of the formulations was calculated as zero and, therefore, it was concluded that they were non-irritants.

Mosquitocidal factor: The mosquitocidal factors of the bacterial strain, VCRC B474, consisted of three proteins with the mw of 27, 67 and 125 kDa (Fig. 1.7), which are similar to those found in *B. thuringiensis* var *israelensis*. The characterization of the mosquitocidal factors of the other two strains VCRC B469 & VCRC B471 is in progress.

Fig. 1.6. Amplified 16s rRNA gene of VCRC B469, & 471 & 474 (Lanes M - 100bp ladder, A - B469; B - B471; C - B474; D - Control)

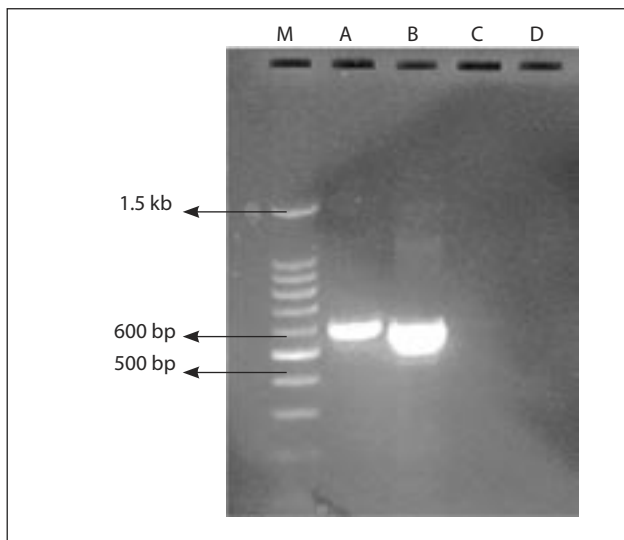
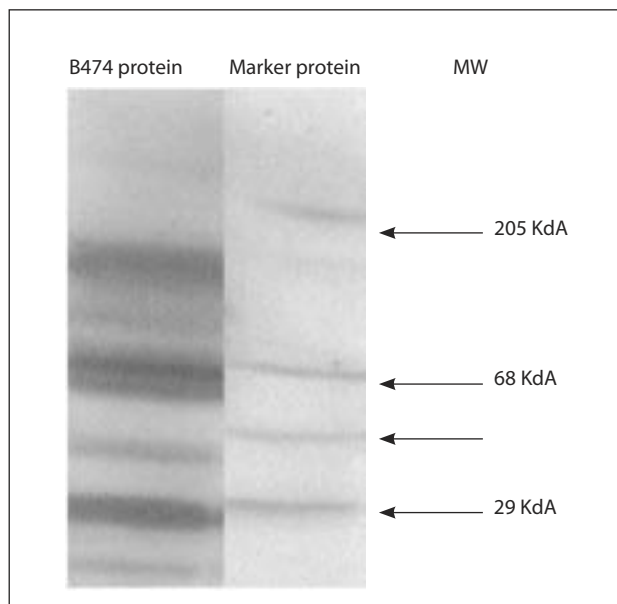


Fig. 1.7. Mosquitocidal factors of VCRC B474 as observed through SDS-PAGE



1.11. Cost-Effective Technology for Culture and Production of Bacillus sphaericus and Bti –Biopesticides for Biological Control of Vector Mosquitoes (S. Poopathi; EM 0203 PD; Duration: 3 years, Dec 2002 – Nov 2005)

The report of the project relates to an economical process for producing bio-insecticides. More particularly, the findings relate to the production of *Bacillus sphaericus* (*Bs*) and *B. thuringiensis* serovar *israelensis* (*Bti*) - based biopesticides utilizing cheap and locally available biological

waste material through simple fermentation technology.

The bacterial culture media preparation and purification of crystal toxins were mentioned earlier (VCRC AR-2003). The crystal toxins from *Bs* and *Bti* produced from media during bacterial growth were analyzed during 2004.

The protein profile of *Bs* and *Bti* produced from new culture media was analyzed by SDS-PAGE and the results were compared (Figs. 1.8

Fig. 1.8. Expression of *B.sphaericus* binary toxins produced from bird feather extract

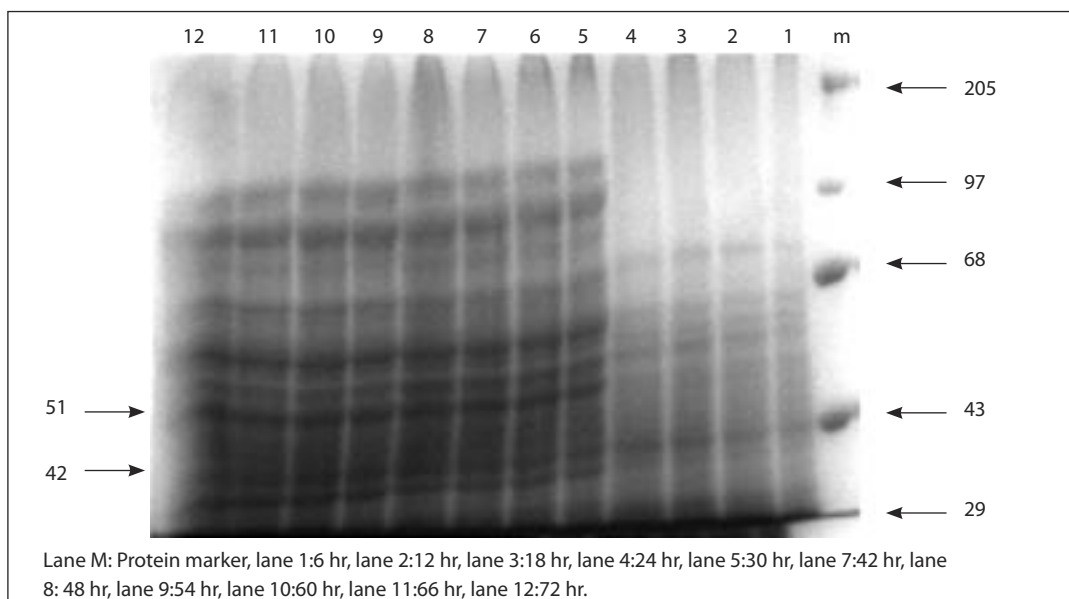
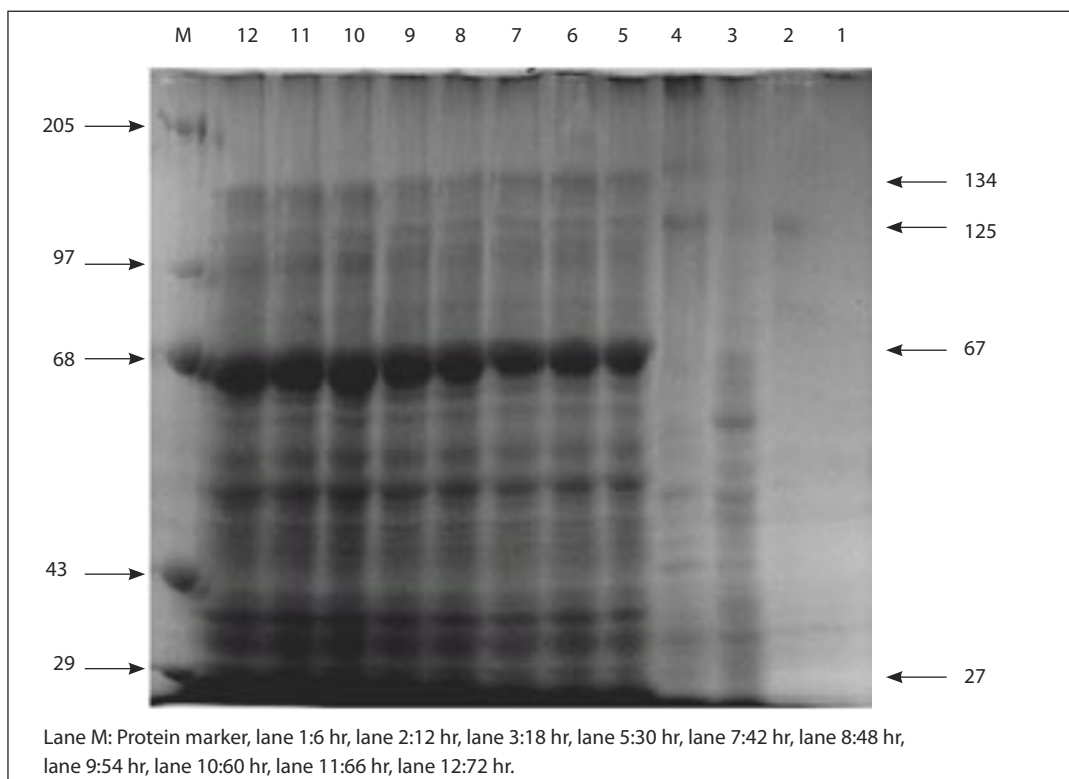


Fig. 1.9 Expression of Bti multiple toxins produced from bird feather extract



& 1.9). The major polypeptides present in the parasporal crystal proteins of *Bs* and *Bti* (*Bs*: 51 and 42 kDa and *Bti*: 134, 125, 67 and 27 kDa proteins) produced from bird feather extract medium was indicative of good expression of toxins during the period of 36 to 72 hours. But the toxins were not produced between 6 and 30 hours. The comparative toxicities of *Bs* and *Bti* produced from the culture media

viz., NYSM and feather extract against *Culex quinquefasciatus* are analyzed. The LC_{50} values were similar for *Bs*, when produced in both the media. Also, toxicity of *Bti* was higher than that of *Bs* and the toxicity was similar when produced in both the culture media. Cost analysis for production of bio-pesticides from feather extract shows that it is much more economical than NYSM.