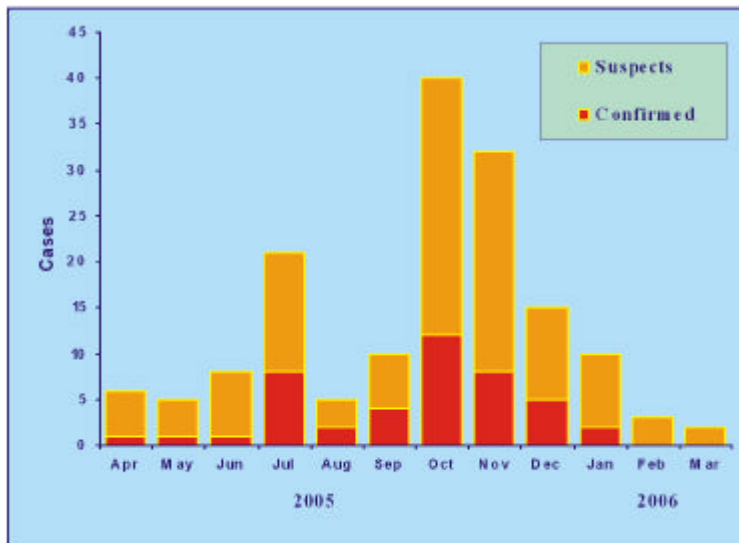


# 1. LEPTOSPIROSIS

## 1.1. Leptospirosis surveillance based at a rural primary health centre, South Andaman

A hospital based surveillance of leptospirosis is being carried out based at a primary health centre (PHC) in South Andaman catering to a population of about 23,000 people living outside the municipal area of Port Blair. Patients attending hospital are being selected based on a clinical criteria, which include acute onset of fever, headache and body aches associated with jaundice, oliguria, cough, breathlessness and haemoptysis, severe muscle tenderness or any bleeding tendency including subconjunctival haemorrhage. Blood samples were collected at the time of reporting and attempts were made to collect a second sample 10-14 days after the collection of the first sample. Acute samples were inoculated into EMJH medium for isolation of leptospires.

Microscopic agglutination test (MAT) was performed on acute samples and convalescent samples. Latex agglutination test was performed on the acute samples for a quick diagnosis for initiating specific therapy. The diagnostic criteria was a four-fold rise in titre with a titre of at least 1 in 80 in the second sample or sero-conversion in MAT in the case of paired samples and a titre of at least 1 in 80 on acute samples in the case of single samples or isolation of leptospires from blood samples. Demographic data was collected by interviewing the patients and the clinical data by abstracting the case records and by clinical examination. Population details were collected from the list of residents maintained by the Andaman and Nicobar Administration and downloadable at their site (<http://andaman.nic.in>). Age and sex specific



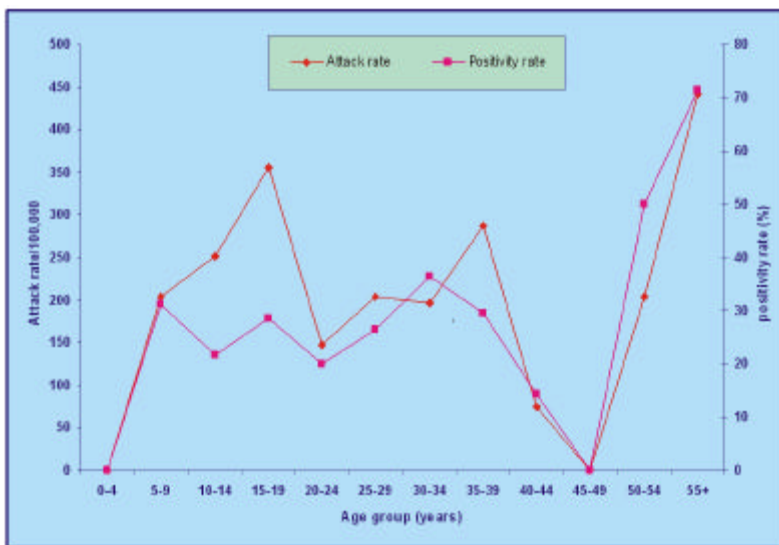
*Fig. 1. 1. Distribution of suspected and confirmed cases of leptospirosis attending Manglutan PHC by month*

positivity ratios and attack rates were calculated. Association between common symptoms and leptospirosis was tested by univariate analysis.

A total of 157 patients were identified during the reporting period. Latex agglutination test was positive on the acute samples of 35 patients. Paired samples could be collected from only 37 patients. Based on the diagnostic criteria described above, a total of 44 patients were diagnosed to have leptospiral infection giving an overall positivity ratio or predictive value of the clinical diagnosis equal to 28%. Amongst the 157 suspected patients 91 were males and out of them 25 were confirmed to have leptospiral infection (positivity ratio 27.5%). Among the 66 female suspected cases leptospiral infection was confirmed in 19 (positivity ratio 28.8%). There was a substantial difference in the mean age of confirmed male patients (23 years) and confirmed female patients (30 years). However, the difference was not statistically significant ( $t=1.391, p=0.171$ ).

The distribution of suspected cases and confirmed cases by month is shown in fig 1.1. There were two peaks in the distribution of suspected and confirmed cases, the first in the month of July and the second in October, which is the pattern usually seen in this area.

As per the list of residents maintained by the Andaman and Nicobar Administration the total population in the villages served by Manglutan PHC is 23,259 and 44 cases in a year among them give an overall attack rate of 189.2/100,000 population. The attack rates among males was 193.9/100,000 and that among the females was 181.6%. The difference between these is not statistically significant ( $\chi^2=0.05, p=0.83$ ). Age-specific attack rate and positivity ratios are shown in figure 1.2. Children below 5 years of age were not affected. The overall trend shows an increase in attack rates with age peaking in early adulthood and then a decline. However, there is an apparent second peak in the older adults aged above 54 years. The reason for this is not understood.



*Fig. 1. 2. Age specific attack rates and positivity ratios of leptospirosis in rural areas serviced by Manglutan PHC during 2005 - 06*

For the purpose of assessing the association between common symptoms and leptospirosis, some of the cases and controls identified outside the study period were also used. There were a total of 88 cases. The results of univariate analysis is summarized in table 1.1. Cough is the only symptom that is statistically significantly associated with leptospirosis. Haemoptysis, haemetemesis and diarrhea showed a positive association with leptospirosis, but the

statistical significance of these was in borderline range.

Among the reactive serovars in MAT, Icterohaemorrhagiae (15%) was the commonest followed by Grippityphosa (6%), Hebdomadis (3%) and Autumnalis (2%). Two leptospires were isolated during the study period. Serological and molecular characterizations of the isolates are underway.

*Table 1.1. Results of univariate analysis with various common symptoms as independent variables and leptospirosis as dependent variable*

<b>SYMPTOM</b>	<b>OR</b>	<b>95% CI</b>	<b>?<sup>?</sup>*</b>	<b>p</b>
Chills	1.61	0.87, 3.01	2.64	0.104
Rigors	1.37	0.58, 3.11	0.64	0.422
Back Ache	1.32	0.69, 2.51	0.81	0.368
Headache	1.60	0.78, 3.34	1.90	0.168
Cough	2.11	1.11, 4.03	6.23	0.0126
Hamoptysis	3.89	0.76, 17.63	4.27	0.0387
Breathlessness	1.13	0.37, 3.21	0.06	0.810
Vomiting	1.21	0.57, 2.51	0.29	0.590
Abdominal pain	0.64	0.19, 1.97	0.69	0.406
Haemetemesis	5.97	0.67, 45.89	4.80	0.0285
Diarrhoea	3.34	0.83, 12.07	4.47	0.0345

\* Uncorrected ?<sup>?</sup>

## 1.2. Leptospiral carrier rate among animals slaughtered in Port Blair

A slaughter-house based study was undertaken with the objective of understanding the distribution patterns of the different leptospiral isolates in various slaughtered animals such as cattle and goats and understanding the serological and molecular characteristics of these isolates. A total of 188 cattle

and 202 goat serum and kidney samples were collected from a slaughterhouse in Port Blair.

Among the 184 serum samples collected from cattle, 63 (34%) showed leptospiral antibodies and among the 202 serum samples collected from goats, 58 (29%)

showed leptospiral antibodies. The common reactive serovars in MAT on cattle and goat serum samples is shown in fig. 1.3. The reactive serovars were similar in the case of cattle as well as goats. Serovar Ballum

did not react with any of the serum samples from cattle. Among cattle *Icterohaemorrhagiae* (37%) was the commonest serovar followed *Hebdomadis* (17%) and *Grippityphosa* (16%). Among goats

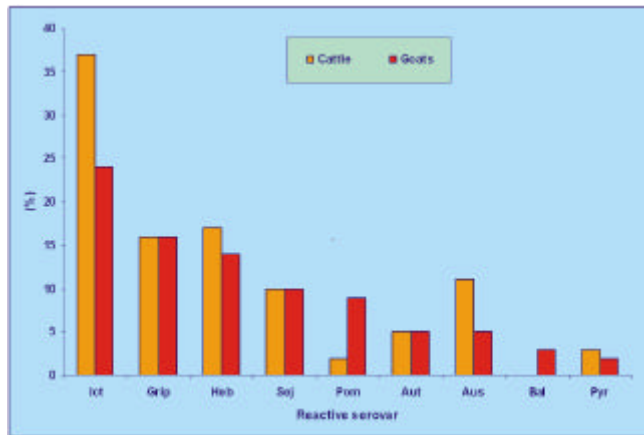


Fig. 1.3. Reactive serovars in MAT in cattle and goat serum samples

Table 1.2. Distribution of slaughtered animals from whom samples were collected by the area from where they were brought to slaughter house

Place	Cattle	Goats	Total
Diglipur	30	86	116
Mayabunder	0	35	35
Billiground	10	0	10
Rangat	25	10	35
Baratang	0	25	25
Neil Island	0	20	20
Calicut	50	0	50
Wandoor	13	0	13
Chouldari	15	0	15
Tusnabad	10	0	10
Faraargunj	10	0	10
Jirkatang	20	0	20
Wimbergunj	5	0	5
Mecca Pahad	0	10	10
Tirur	0	12	12
Port Blair	0	4	4
<b>Total</b>	<b>188</b>	<b>202</b>	<b>390</b>

Icterohaemorrhagiae (24%) was the commonest serovar followed by Grippotyphosa (16%) and Hebdomadis(14%).Mixed equals were observed in 7 samples collected from goats. The reaction were mainly among Icterohaemorrhagiae and Grippotyphosa and Autumnalis and Icterohaemorrhagiae. The infecting serovars both in the case of goats and cattle as per MAT titres were the same as those observed in human infections also.

These cattle and goats were brought to the slaughter house near Port Blair from different places in South Andaman and nearby islands. The details of areas

from where these cattle and goats were brought are given in Table 1.2. Analysis of the serological tests with sera of animals from different areas showed the prevalence of leptospirosis in all the above-mentioned areas from where the animals were brought for slaughter with a few exceptions such as Neil Island and Baratang.

There were 4 cattle isolates from kidney samples which were brought from Calicut, a place located near Port Blair city. All the four isolates belonged to serovar Icterohaemorrhagiae of serogroup Icterohaemorrhagiae.

### 1.3. Role of polymerase chain reaction (PCR) in diagnosis of leptospirosis in urine samples of human and animals

A study was undertaken with the objective of evaluating the role of urine PCR in diagnosis of leptospirosis. A total of 82-paired serum samples were collected from Manglutan PHC from suspected human cases of leptospirosis. After a week of 1st serum samples collection, 2nd serum samples were collected along with urine samples of the same patients.

Leptospiral antibody was tested using MAT. 8 (10%) serum samples showed leptospiral antibody.

Icterohaemorrhagiae was the commonest serovar (50%) followed by Grippotyphosa (25%) and Australis (13%). Thirteen (16%) urine samples were positive by PCR. 3 (4%) samples were both PCR and MAT positive. 10 (12%) samples were positive by PCR but negative by MAT and 5 were MAT positive and were negative by PCR. The correlation between serum MAT results and urine PCR results is shown in table 1.2. Total negatives both by PCR and MAT were 64 (78%) samples.

*Table 1.3. Correlation between serum MAT results and urine PCR results among suspected patients of leptospirosis attending a PHC in South Andaman*

		Serum MAT Results		
		Pos	Neg	Total
Urine PCR	Pos	3	10	13
	Neg	5	64	69
	Total	8	74	82

## 1.4. Application of PCR in early diagnosis of leptospirosis.

Early diagnosis of leptospirosis has an important role for better treatment and prevention of the disease. An attempt has been made to find the correlation between PCR, MAT, isolation and Lepto-Dipstick as diagnostic tools during the acute stage of the disease. Two hundred seventy six patients in whom all the results were available were reconsidered for this study. Out of these cases, 72 patients were diagnosed as leptospirosis cases either serologically or microbiologically. Among these 72 patients 68 patients were diagnosed serologically. Seventy-eight cases were identified as positive by PCR (Table 1.3). Forty-five cases were Dipstick positive, and 26 were isolate positive. PCR and standard criteria were

positive in 68 cases and in 194 patients both were negative (Table 1.4). The sensitivity of PCR when compared to standard criteria was 94.4% and specificity 95.1%. The sensitivity of PCR when compared to isolation was 92.3%, while Dipstick showed low sensitivity of 37.5% compared to standard criteria. PCR was able to detect 8 culture positive cases that were negative by paired MAT and also able to detect 4 culture positive cases that were negative by MAT on single sample. However, though PCR is expensive and requires expertise, its use in clinical samples particularly in outbreak situations where quick diagnosis is required is indispensable.

*Table 1.3. PCR Vs Std criteria and PCR indices*

		Standard criteria		
		Pos	Neg	Total
PCR	Pos	68	10	78
	Neg	4	194	198
	Total	<b>72</b>	<b>204</b>	<b>276</b>
<b>Sensitivity</b>		94.4%(85.7,98.2)		
<b>Specificity</b>		95.1%(90.1,97.5)		
<b>PPV</b>		87.2%(77.2,93.3)		
<b>NPV</b>		98.0%(94.6,99.6)		
<b>%Agreement</b>		94.9%		
<b>Kappa</b>		0.8719		

*Table 1.4. Dipstick Vs Std criteria and dipstick indices*

		Standard criteria		
		Pos	Neg	Total
PCR	Pos	27	18	45
	Neg	45	186	231
	Total	<b>72</b>	<b>204</b>	<b>276</b>
<b>Sensitivity</b>		37.5%(26.6,49.7)		
<b>Specificity</b>		91.2%(86.2,94.5)		
<b>PPV</b>		60.0%(44.4,73.9)		
<b>NPV</b>		80.5%(74.7,85.3)		
<b>%Agreement</b>		77.3%%		
<b>Kappa</b>		0.3264		

## 1.5. Molecular characterization of leptospiral isolates using Restriction Fragment Length Polymorphism (RFLP) techniques

Polymorphisms within segments of the 16s and 23s rRNA genes were studied with the objective of determining the utility of PCR-RFLP technique in characterization and identification of the different species/strains of *Leptospira*.

Twenty six reference strains belonging to eight different genospecies of *Leptospira* and forty one isolates were included in the study. A 631 bp segment of the 16s rRNA gene and a 2300 bp segment of the 23s rRNA gene were amplified by PCR following published protocols. Restriction patterns were generated for each of the reference strains and isolates on these segments with twelve restriction enzymes.

The 631 bp amplified product of 16s rRNA region when digested with restriction enzyme *HinfI* showed 3 types of patterns among the 8 genospecies studied. When the same products were digested with *DdeI* restriction enzyme, it showed better discrimination between the different genospecies and was found to differentiate between strains within individual genospecies. Among ten different strains of *L. interrogans*, it differentiated two strains Ballico and Swart that showed different patterns from the rest. Two strains Patoc 1 and Andamana of the genospecies *L. biflexa* showed different *DdeI* restriction patterns. Among four strains of *L. kirschneri*, one strain Butembo showed patterns different from the rest. Both the strains Panama and LSU1945 of *L. noguchi* showed difference. The lone strain of *L. meyeri* showed a unique pattern that did not match with other strains studied. With other enzymes, however, no further discrimination was found within this 631bp region.

When the 2300bp segment of 23s rRNA gene of the reference strains belonging to 8 genospecies were digested with *DdeI* restriction enzyme, strain Ballico could be differentiated from other nine strains of *L. interrogans*. Three strains of *L. kirschneri* viz. Butembo, Cyanopteri and Moskovash showed different patterns. Two strains of *L. noguchi* also showed different patterns and could be distinguished from each other and from the rest of the genospecies. *L. welii* showed a unique pattern in this region. The two strains of *L. biflexa* also showed different patterns. However, *DdeI* enzyme digestion of 2300bp amplicon did not differentiate among the three strains of *L. santarosai*.

The same technique was applied on the isolates of *Leptospira* from the Andamans and from mainland India. It was found that the isolates from Andaman Islands did not possess much polymorphism in the 16s region. These isolates however showed 3 types of patterns in the amplified segment of the 23s rRNA region after digestion with different enzymes.

*HinfI* restriction patterns of mainland isolates revealed 3 different types for isolates from the three states Karnataka, Tamil Nadu and Kerala. The strain Thankachan from Tamil Nadu showed *HinfI*-RFLP patterns that matched with those of the isolates from these islands suggesting possible epidemiological links.

The study revealed that PCR-RFLP of the rRNA genes of *Leptospira* with the enzyme *DdeI* could be of some use for characterization of certain leptospiral genospecies and can aid in molecular epidemiological studies. However, in the absence of proper clustering of strains according to their

genospecies and in absence of capability to discriminate between several genospecies and strains the utility of this technique in species/strain level

identification of leptospire remain limited, at least in the regions studied.

## 1.6. *flaB* PCR based identification of pathogenic and non pathogenic leptospiral isolates

Conventionally leptospire are classified into two species, the pathogenic *Leptospira interrogans* and the saprophytic *L. biflexa*. *L. biflexa* occurs as natural contaminant of surface waters and wet environment. When leptospire are isolated from human, animal or environmental samples, it is necessary to identify whether these belong to pathogenic species or saprophytic species. The conventional methods for this is based on the ability of the organism to grow on specific growth conditions such as at 13°C and in the presence of 8-azaguanine. However, there have been instances where isolates obtained from human blood samples are classified as saprophytic based on these tests. It has been reported that genes coding for several proteins are present only in pathogenic species. Flagellar protein is one among them. We evaluated this gene as a marker of pathogenic status of leptospire.

A total 55 leptospiral isolates were utilized for this study, which were obtained from various sources like human blood (n=42), urine (n=1), rat kidney (n=4), cow urine (n=1) and water bodies (n=3). Isolates recovered from the human specimens were collected from the ailing patients suspected for leptospirosis. All the isolates were identified up to serovar level. Out of the 55 isolates obtained, 22 belonged to Grippityphosa, 7 to Pomona, 5 each to Icterohaemorrhagiae and Canicola, 3 to Australis,

and 1 each to Pyrogenes, Sejroe, Autumnalis and Hebdomadis. The remaining 9 were unclassified.

Conventional techniques like growth at 13°C and 8-azaguanine tests were initially performed to find out the pathogenic/non-pathogenic status of the isolates. The inoculated tubes were incubated up to 21 days and the tubes were observed at 0 days, 7 days, 14 days and at 21 days under dark field microscope. The leptospire observed for each isolate were recorded and the pathogenic status of the isolates were determined in comparison with the pathogenic and non-pathogenic controls. The growth rate observed for five pathogenic and five non-pathogenic isolates in comparison with the controls were plotted in a graph for comparison. The maximum number of leptospire observed for the pathogenic isolates were in the range of 10-150 during the entire period of 21 days and the non-pathogenic isolates showed 400-1020 organisms/field. Based on these analyses, 46 isolates were confirmed as pathogenic and 9 were confirmed as non-pathogenic. None of the non-pathogenic isolates reacted with any of the groups sera of the pathogenic serovars representing 23 serogroups and were categorized in to un-classified isolates. All these Pathogenic and Non-pathogenic leptospire were amplified using I specific *flaB* primers. PCR could amplify all the pathogenic leptospire and did not amplify any non-pathogenic leptospire.

## 1.7. Identification, characterization and role of proteinases during the pathogenesis of leptospirosis

Leptospirosis, a zoonotic disease, is a worldwide health problem. The pathogenesis of leptospirosis is poorly understood. Recent developments in the genomics of leptospires, the causative organism, reveal the presence of some proteinase genes. Metalloproteinases are zinc-containing endopeptidases that degrade extracellular matrix proteins during tissue morphogenesis and remodeling in wound healing and are associated with tumor angiogenesis, invasion and metastasis, arthritis and atherosclerosis in eukaryotes. Bacterial pathogens synthesize and secrete a wide array of proteinases, of which the most common are metalloproteinases. These enzymes work against host proteinase cascades, cytokine networks, extra-cellular matrix components and host enzyme inhibitor interactions. Until now not much studies have carried out in leptospirosis on this subject and not much information is available on metalloproteinases in *Leptospira*.

The study involves the identification of proteinases during the pathogenesis of leptospirosis and finding out their role in host pathogen interaction. The pathogen and the host cells will be stimulated to express their proteinases by exposing to simulated environment of infection. The proteinases will be characterized on the basis of its pH, requirement of different metal ions and effect of proteinase

inhibitors. Similarly human mononuclear cells that are primary to immune response will be challenged with leptospires or its antigen to find the release of host proteinase and cytokines. The knowledge of the suitable environment, which stimulates the pathogen to produce proteinases, will be used to study the invasion of leptospira into host cell. Different inhibitors of proteinases will be used to assess the importance of those proteinases in the process of invasion.

*Leptospira* were cultured in EMJH medium containing 1% gelatin. Cultures were centrifuged at 15,000 rpm at 4°C. The pellet was dissolved in 50 mM Tris-Cl, pH 7.2 containing 0.5% Deoxycholate and 0.75M 6-Aminocaproic acid and centrifuged again. Proteinase activity in the supernatant was assayed by zymography where the corresponding bands will be observed as cleared zones in coomassie stained 1% gelatin containing gel in which the electrophoresis of the protein was carried out.

The main findings of preliminary studies include (1) identification of a gelatinase activity in a protein with molecular mass ~70 kDa, (2) identification of this activity in 15 different serovars of *Leptospira*, (3) establishment of calcium ion requirement for the activity of the identified gelatinase, and (4) establishment of EDTA inhibition of this activity. Further characterization of the enzyme is underway.

## 1.8. Cloning and expression of leptospiral proteins OmpL1 and LipL41

Leptospiral genes encoding for their corresponding proteins were cloned in prokaryotic expression

systems and they were expressed and purified. The purified recombinant proteins were evaluated for the

diagnosis of leptospirosis using ELISA. The leptospiral genes *OmpL1* and *LipL41* coding for the leptospiral proteins OmpL1 and LipL41 were cloned in plasmid vectors pRSETA and pET15B. For cloning the fragments the gene containing the DNA fragments were amplified using specific primers and

20 mM. The optimal expression is even achieved at the minimal concentration 0.1mM. After the expression, the recombinant proteins were purified using affinity chromatography with Ni<sup>2+</sup> agarose columns. The purity of eluted *His6* fusion proteins was assessed by gel electrophoresis. Proteins were

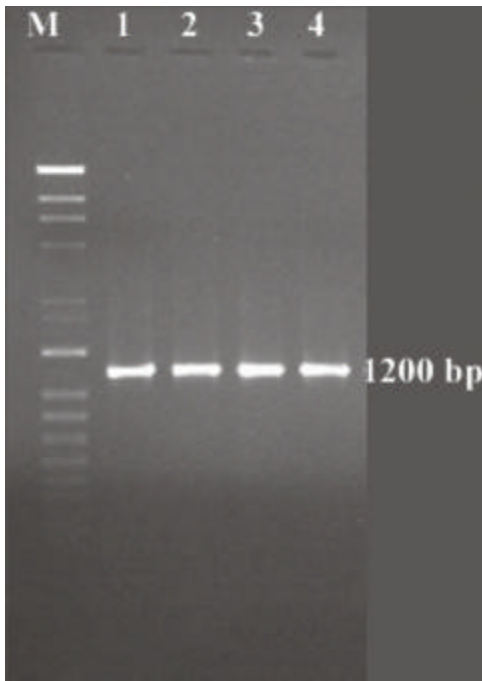


Fig. 1. 4. PCR based confirmation (*M-Lambda/Hind III*, Lanes 1-4-*LipL41+pET15b* clones)

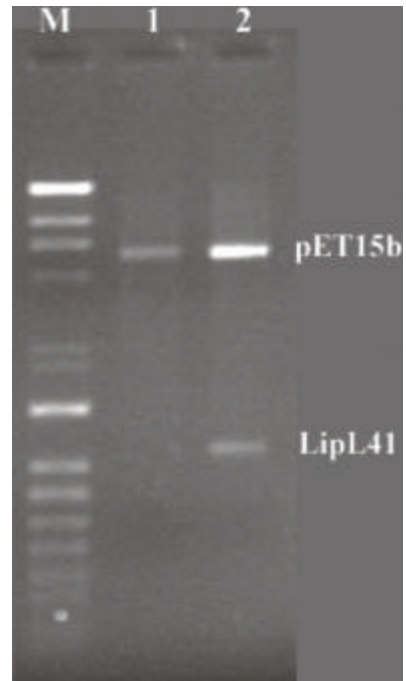


Fig. 1. 5. *LipL41* and *pET15b* (*Nco I, Xho I*) (*M-Lambda/Hind III*, lane 1-*pET15b*, 2-*LipL41+pET15b*)

the *OmpL1* and pRSETA were digested with *HindIII* and *EcoRI*, then they were ligated and transformed into *E.coli* BL21 (DE3). The same way the fragment containing LipL41 was amplified and *LipL41* and pET15B were digested with *NcoI* and *XhoI*. Then they were ligated and transformed into *E.coli* JM109 (DE3). The clones of four interest were selected based on antibiotic markers. Then the clones were expressed at different IPTG concentrations like 0.1 to

dialyzed overnight against phosphate-buffered saline (PBS)-10% glycerol-0.025% sodium azide-0.1% Triton X-100-0.1%. After dialysis, the protein concentration was determined with bicinchoninic acid. Then the various concentrations of these proteins were coated in ELISA plates. Using known positive sera sample the optimal concentration to be utilized was determined as 30ng/well. Flat bottom ELISA plates were coated with 30ng/well of the

recombinant proteins individually and evaluated using known positive and negative sera samples. Based on the negative sera samples the mean+1SD were taken as the cutoff value for the determination

of the titre and the results showed a sensitivity of 92.3%, specificity 86.7%, PPV 85.7%, NPV 92.8% for OmpL1 and a sensitivity of 96.2%, specificity 83.3%, PPV 83.3%, NPV 96.2% for LipL41.



**HEALTH AWARENESS CAMP ON LEPTOSPIROSIS AT GUPTA PARA VILLAGE WHERE LEPTOSPIROSIS IS ENDEMIC**

