

Application of recombinant Leptospiral outer  
membrane protein  
in  
ELISA-based serodiagnosis

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# Background to study



## Leptospirosis :

Caused by bacteria of the genus *Leptospira*

Humans infected by contacting to infected urine of carrier animals

Common in temperate (0.1-10/100000)  
or tropical climates (10-100/100000)

# Clinical manifestation

- Incubation periods: 5-14 days
- Subclinical
- Symptomatic 2 phase: **biphasic fever**
  1. **Leptospiremic phase**  
fever, headache, myalgia, conjunctival suffusion
  2. **Immune phase (Weil disease)**  
liver/renal fail, aseptic meningitis



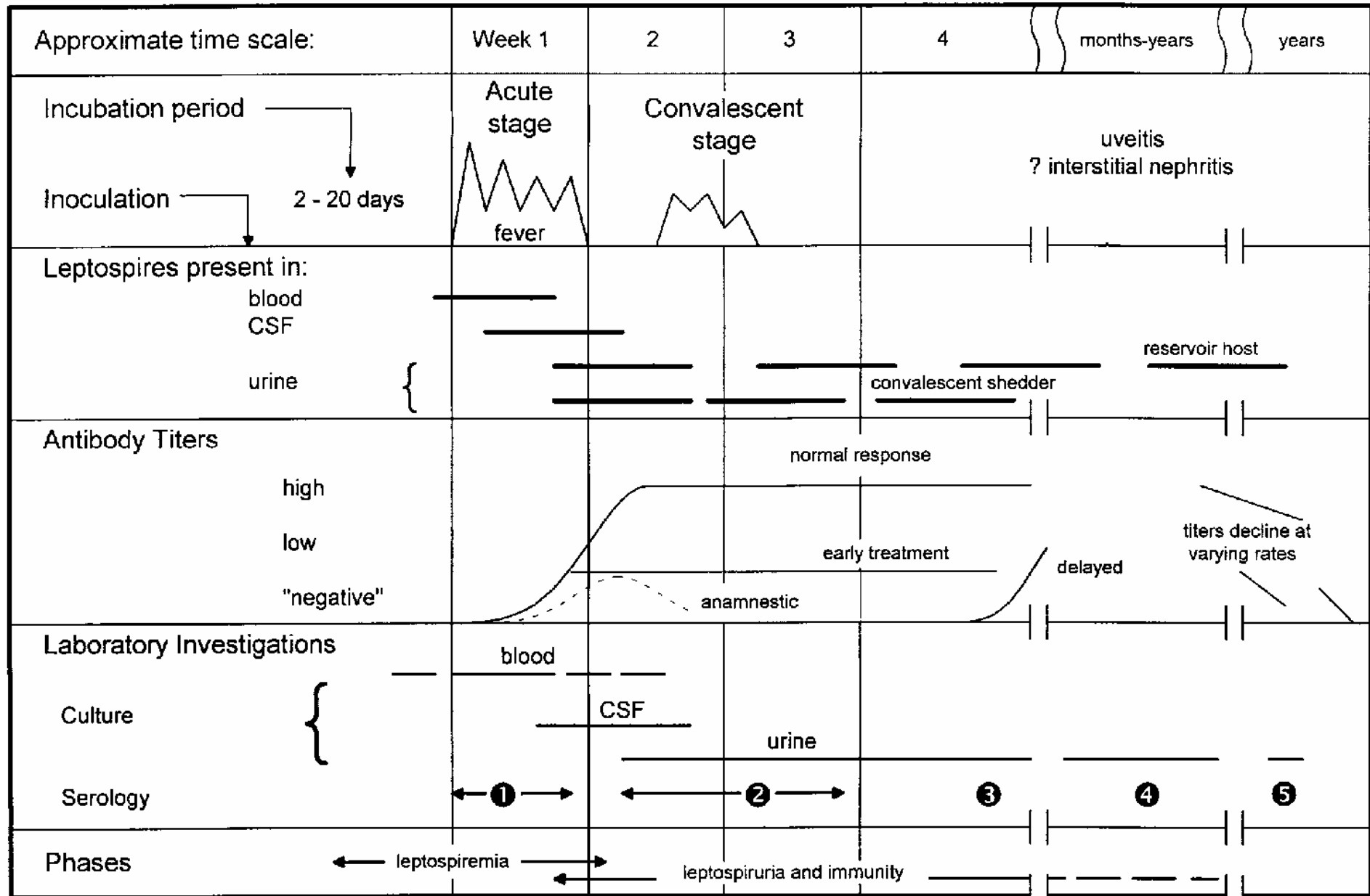
# STANDARD CRITERIA FOR DIAGNOSIS

Patients fulfilling any of the following criteria were considered as confirmed cases of leptospirosis;

- Positive blood culture
- Seroconversion in MAT with a minimum titer of 1:100 in the second sample
- Four fold rise in titer in MAT

Microagglutination test (MAT) is inadequate for rapid case identification, as it can only be performed in reference laboratory.





Leptospiremia



Immune phase

# Benefit of recombinant protein

- ◆ The recombinant leptospiral proteins are possible to be express and purify those fusion proteins in a form suitable for diagnostic formats such as ELISA assay, Western blot.
- ◆ Recombinant protein-based serologic tests achieve high sensitivity and specificity because of the high concentration of immunoreactive antigens were used in assays and the lack of nonspecific moieties presented in whole-cell preparations.



# Recent application of recombinant protein in human Leptospirosis

- Flannery et al.( 2001) evaluated 3 recombinant protein; rHsp58, rLipL32, rOmpL1, using IgG-based ELISA.  
rLipL32 had the highest sensitivity; 56% in acute and 94% in convalescent, in comparative to MAT.
- Srimanote et al. (2007) applied rLigA based ELISA for serodiagnosis with specificity greater than 95%, in comparative to MAT.
- Croda et al (2007) employed rLigB in immunoblot assay using both IgG and IgM conjugate to detect acute phase of disease



An ideal test will need to discriminate between leptospirosis and a broad spectrum of diseases that cause acute febrile illnesses and have overlapping clinical presentations.

This study will include the following serum samples;

- Leptospirosis with MAT positive
- Scrub typhus (ST)
- Dengue fever (DHF)
- Melioidosis (melioid)
- Human serum from endemic area (HE)
- Human serum from non-endemic area (HOE)  
MAT were negative



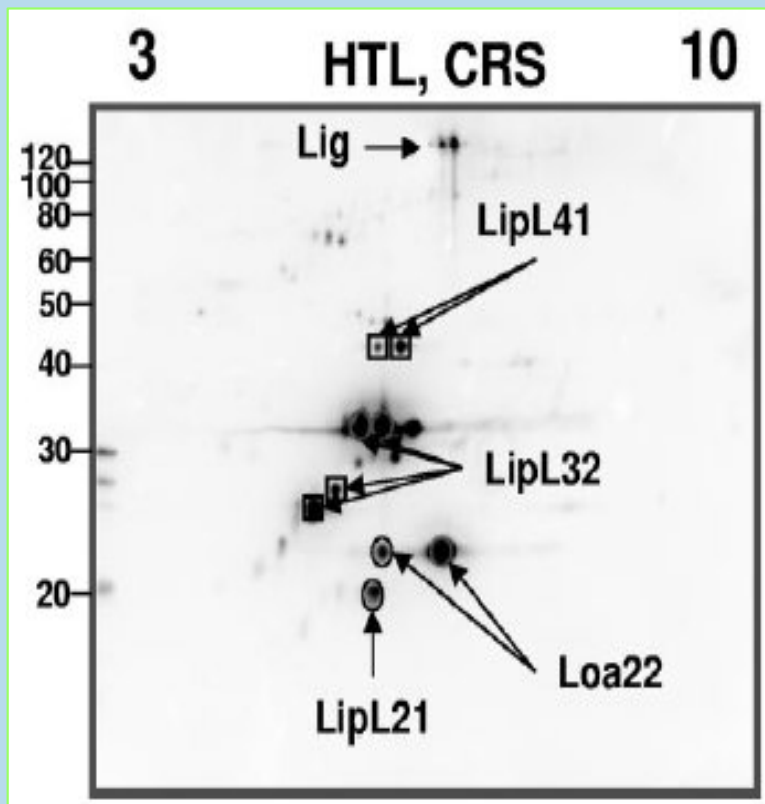


# Characterization of the Outer Membrane Proteome of *Leptospira interrogans* Expressed during Acute Lethal Infection<sup>¶</sup>

Jarlath E. Nally,<sup>1\*</sup> Julian P. Whitelegge,<sup>2,3</sup> Sara Bassilian,<sup>2,3</sup> David R. Blanco,<sup>1</sup> and Michael A. Lovett<sup>1</sup>

*Division of Infectious Diseases, Department of Medicine,<sup>1</sup> The Pasarow Mass Spectrometry Laboratory,<sup>2</sup> and The Jane and Terry Semel Institute for Neuroscience and Human Behavior,<sup>3</sup> David Geffen School of Medicine, University of California Los Angeles, Los Angeles, California*

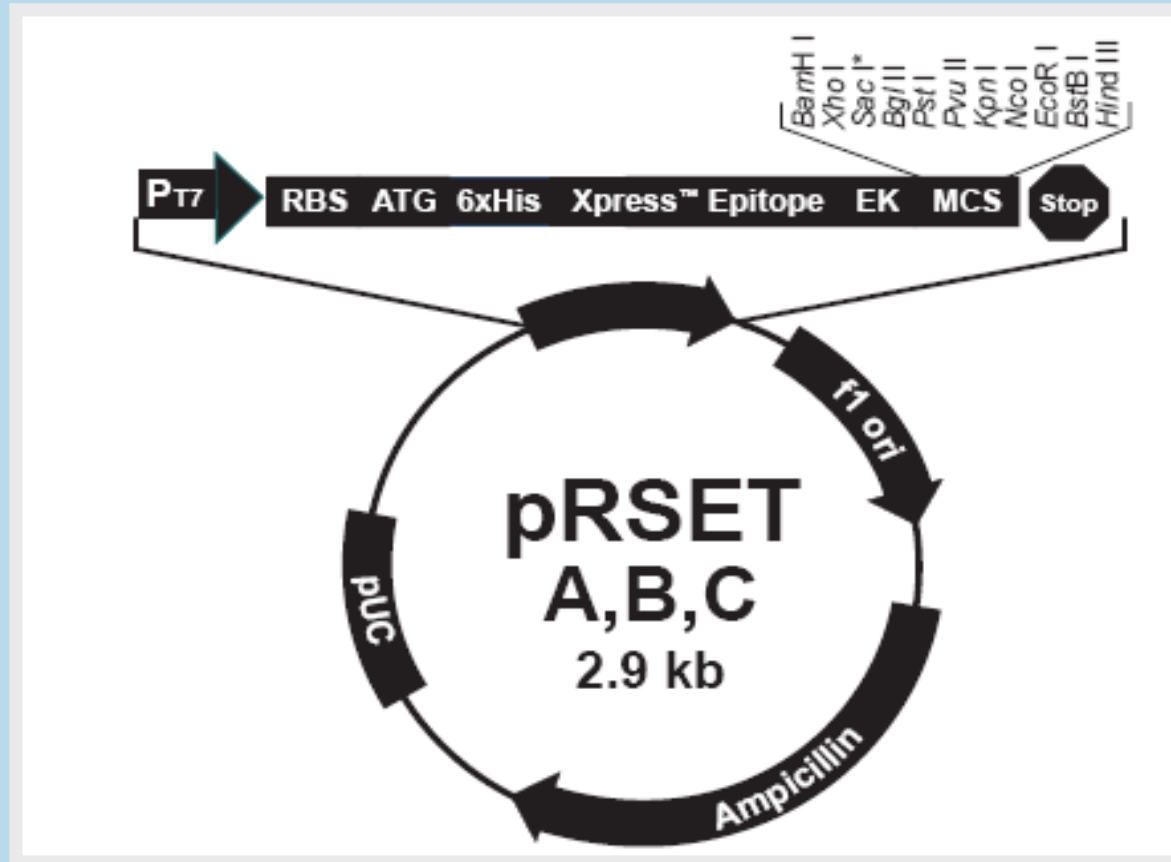
Received 9 May 2006/Returned for modification 1 June 2006/Accepted 30 October 2006



Proteome study revealed the presence of LigA, LipL41, LipL32, LipL21, Loa 22, in host tissue *Leptospira* (HTL) probe with chronic rat serum (CRS)



# pRSET-B Cloning vector



- N-terminal 6x Histidine fusion peptide
- Ampicillin resistance gene

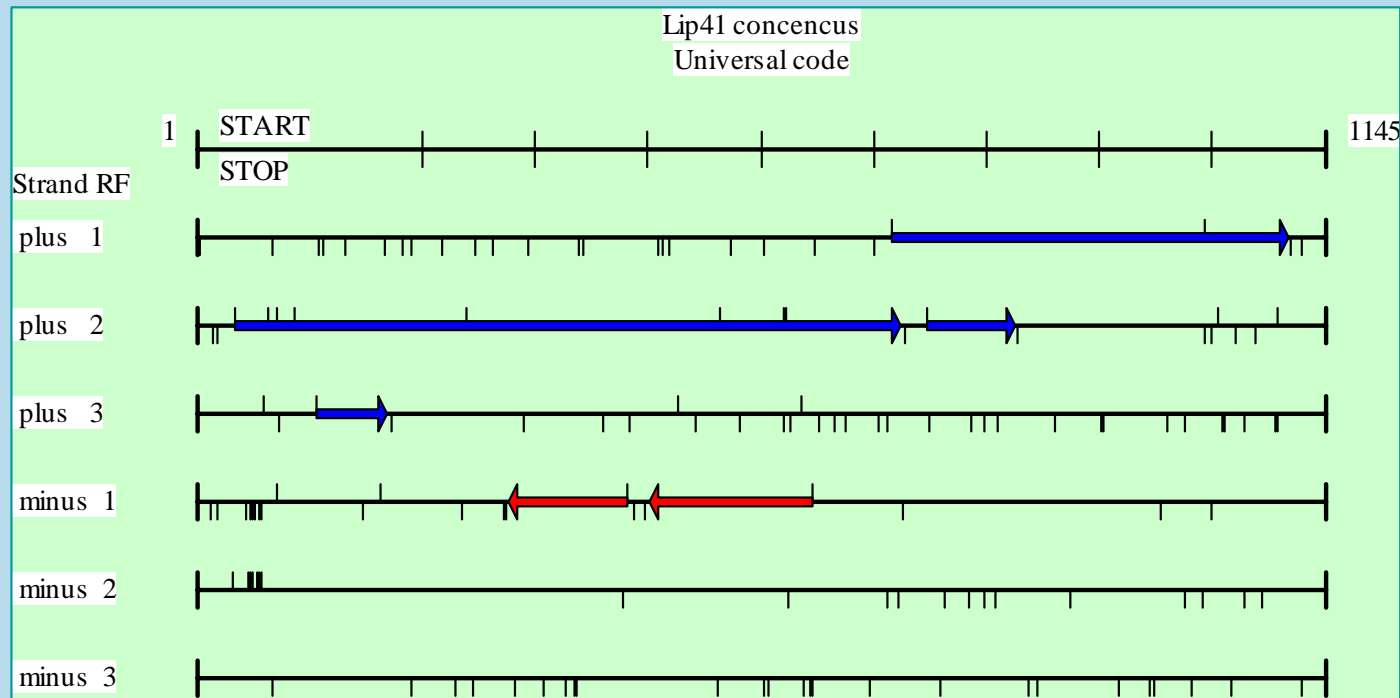


# Prediction of Lipoprotein from *L. interrogans* serovar Copenhageni

Protein	MW; kD	spII score	splip	MW; kD Obtained expressed - protein
LipL41 (Lic12966)	38.93	10.63	probable lipoprotein	27
LipL32 (Lic11352)	29.61	15.73	probable lipoprotein	35
LipL21 (Lic10011)	19.66	21.70	probable lipoprotein	27
Loa22 (Lic10191)	20.91	18.70	not lipoprotein	27



# Translation of LipL41 clone



There is a stop codon within LipL41 clone, so the predicted MW of expressed protein is 24.34 kD.



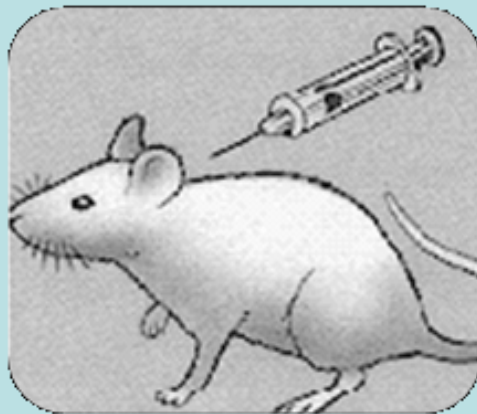
# Validation of Leptospiral recombinant protein

- Prepare the antibody to recombinant protein, and use it to react with native antigen of *Leptospira*
- Determine the reactivity to human serum

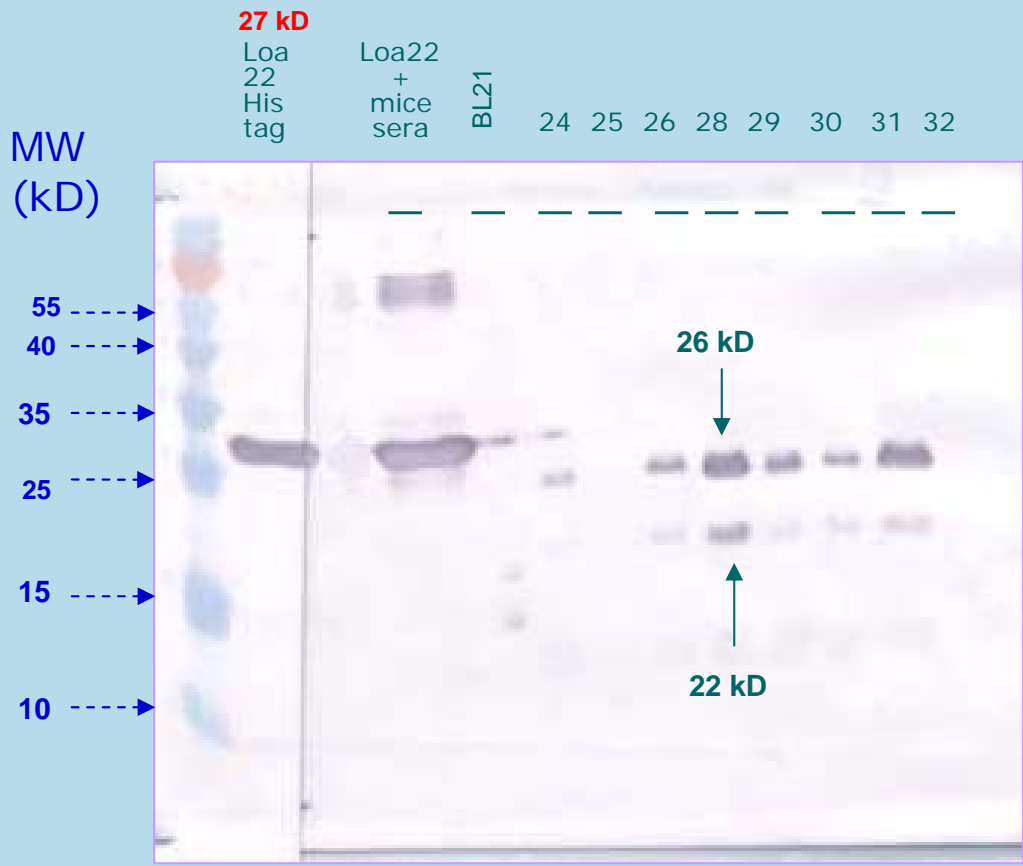


# Mice immunization

- Intraperitoneal route with 5-10  $\mu\text{g}$  with Alum adjuvant per dose.
- Three doses with 2 weeks interval



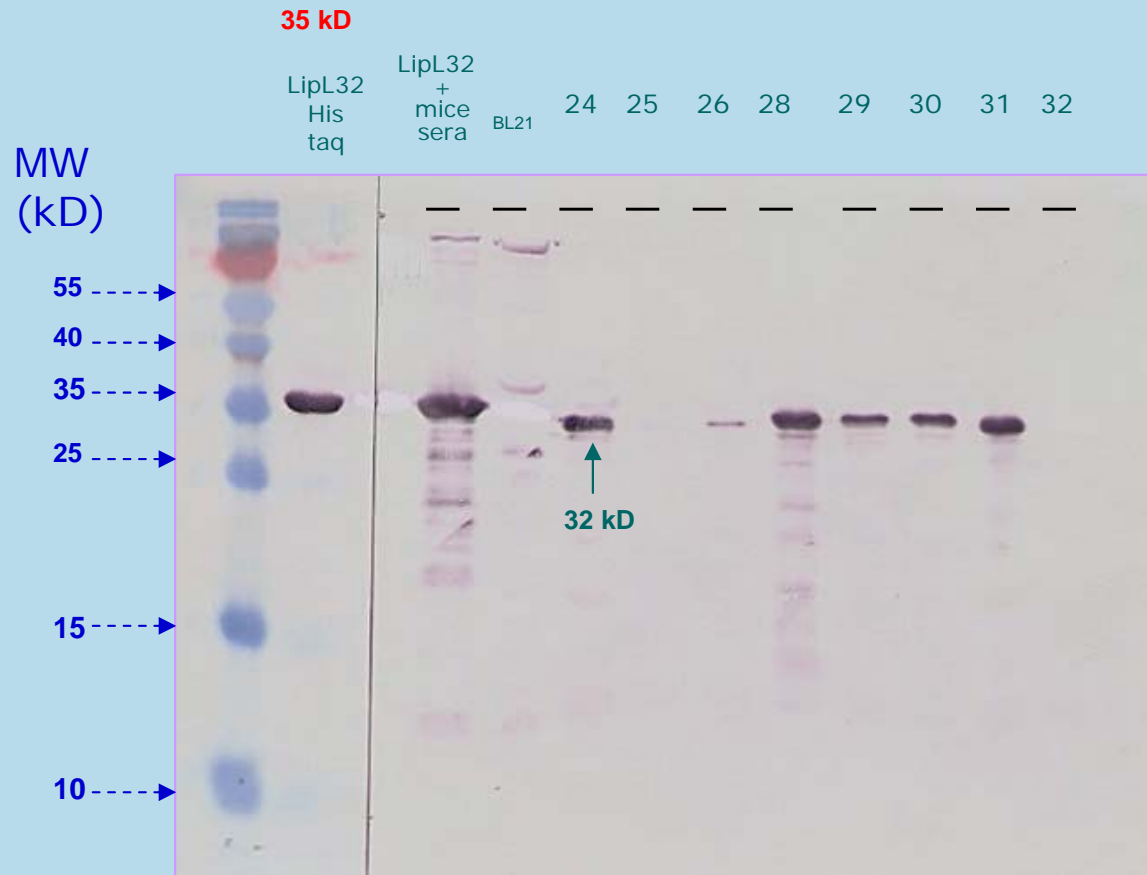
# Reactivity of anti Loa22 to leptospiral lysate panel-2



- Panel of Leptospiral serovar
- 24 New
  - 25 Ranarum
  - 26 Sarmin
  - 28 Mini
  - 29 Cynopteri
  - 30 Louisiana
  - 31 Panama
  - 32 Shermani



# Reactivity of anti LipL32 to leptospiral lysate panel-2



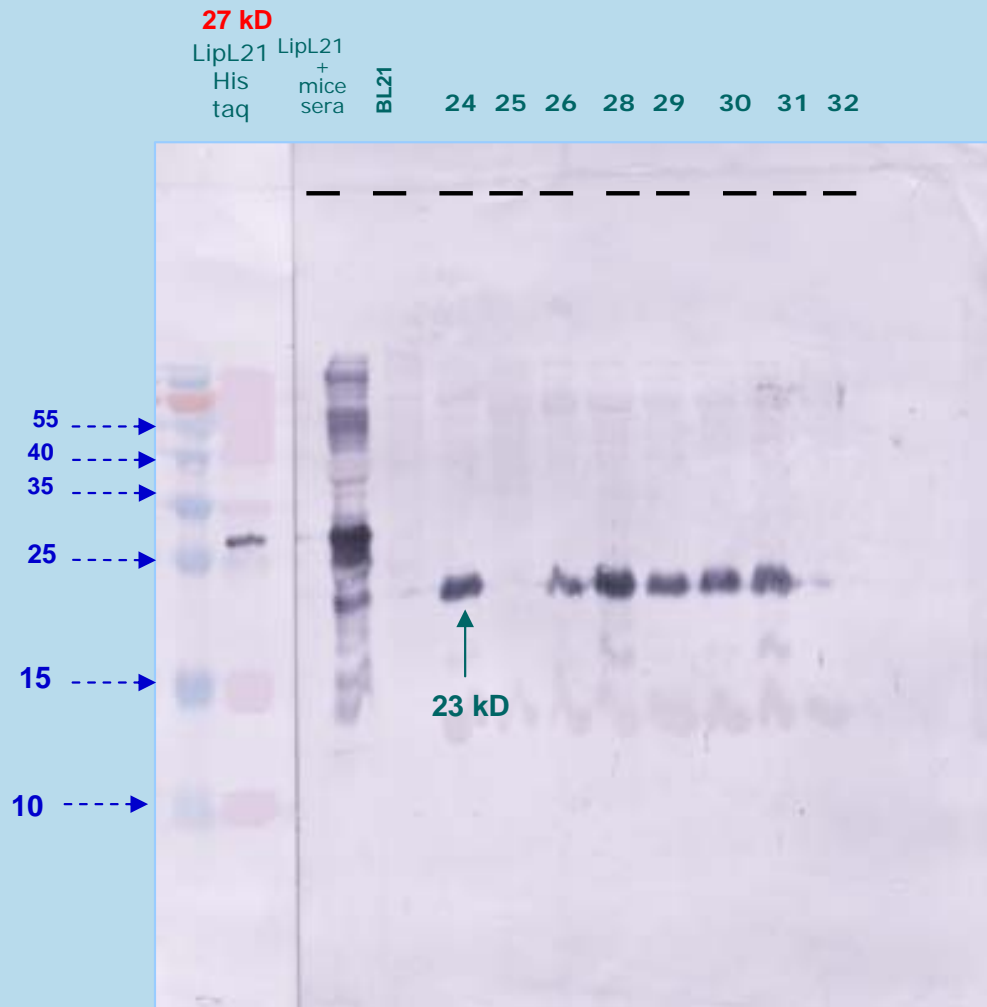
Panel of Leptospiral serovar

24	New
25	Ranarum
26	Sarmin
28	Mini
29	Cynopteri
30	Louisiana
31	Panama
32	Shermani





# Anti LipL21 vs Lepto cell panel 2



Panel of Leptospiral serovar

24 New

25 Ranarum

26 Sarmin

28 Mini

29 Cynopteri

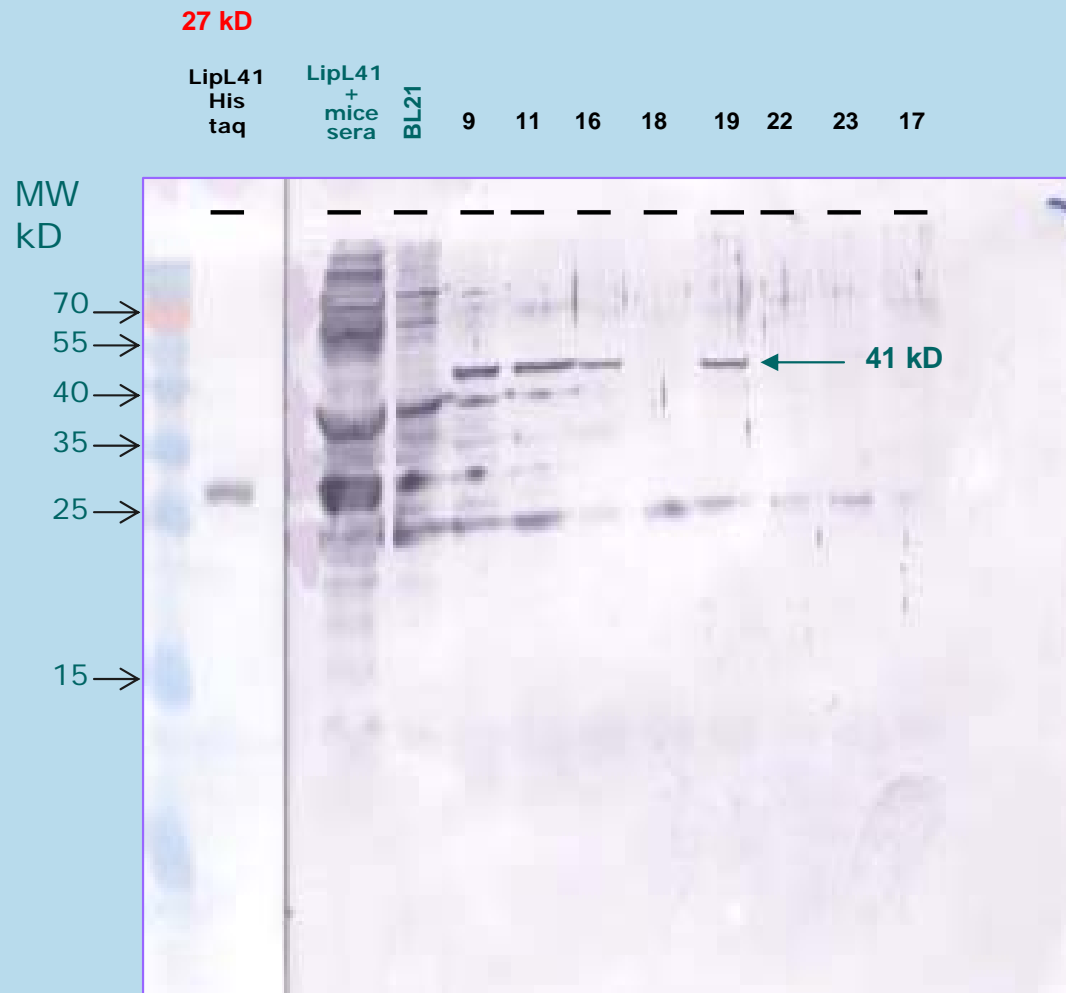
30 Louisiana

31 Panama

32 Shermani



# Anti LipL41 vs Lepto cell panel 1



Panel of Leptospiral serovar

- 9      Copenhageni
- 11     Djasiman
- 16     Javanica
- 18     **Pomona**
- 19     Pyrogenes
- 22     **Sejroe**
- 23     **Wolffi**
- 17     **Patoc**

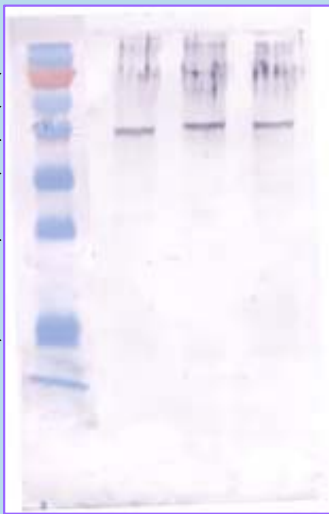
**Negative color**



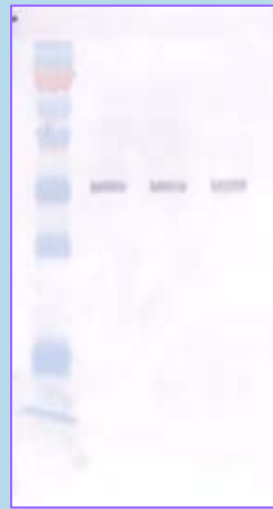
# Reactivity to *L wolffii*

kD

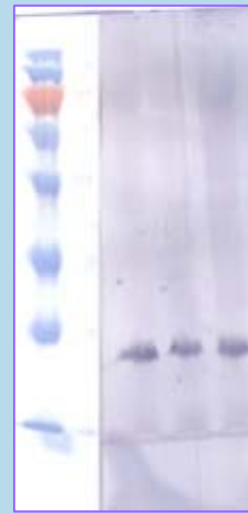
70 →  
55 →  
40 →  
35 →  
25 →  
15 →



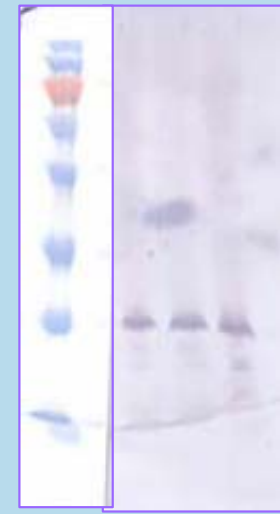
Anti LipL41



Anti LipL32



Anti LipL21



Anti Loa22



## Reactivity of mice anti recombinant protein to Leptospiral whole cell lysate

<i>Leptospira species</i>	Lab no./ serovar	Serogroup	Anti Loa 22	Anti LipL32	Anti LipL21	Anti LipL41
<i>L. interrogans</i>	9 Copenhageni	Icterohaemorrhagiae	+	+	+	+
<i>L. interrogans</i>	11 Djasiman	Djasiman	+	+	+	+
<i>L. borgpetersen</i>	16 Javanica	Javanica	+	+	+	+
<i>L. interrogans</i>	18 Pomona	Pomona	-	-	+	-
<i>L. interrogans</i>	19 Pyrogenes	Pyrogenes	+	+	+	+
<i>L. borgpetersen</i>	22 Sejroe	Sejroe	-	-	+	-
<i>L. interrogans</i>	23 Wolffi	Sejroe	+	-	+	-
<i>L. biflexa</i>	17 Patoc	Patoc	-	-	-	-
<i>L. interrogans</i>	24 New	Autumnalis	+	+	+	+
<i>L. meyeri</i>	25 Ranarum	Ranarum	-	-	-	-
<i>L. weilli</i>	26 Sarmin	Sarmin	+	+	+	-
<i>L. borgpeterseni</i>	28 Mini	Mini	+	+	+	+
<i>L. kirshneri</i>	29 Cynopteri	Cynopteri	+	+	+	+
<i>L. noguchii</i>	30 Saigon	Louisiana	+	+	+	+
<i>L. noguchii</i>	31 Panama	Panama	+	+	+	+
<i>L. santarosai</i>	32 Shermani	Shermani	-	-	-	-
<i>L. wolffii</i>	Khorat	Khorat	+	+	+	+

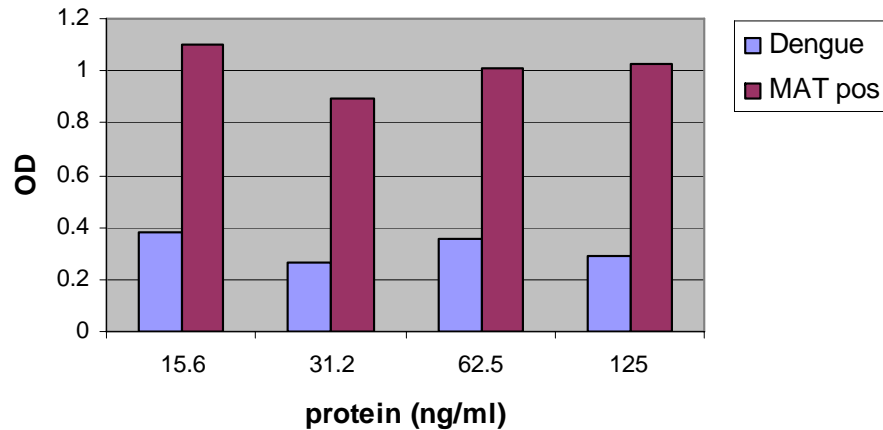


ELISA assay employing Total Igs conjugate HRP  
(predominant IgG class was conjugated to HRP)



# Optimization of ELISA-based assay

### Optimization of rLoa22 antigen conc



The following protein concentration of Ag were used;

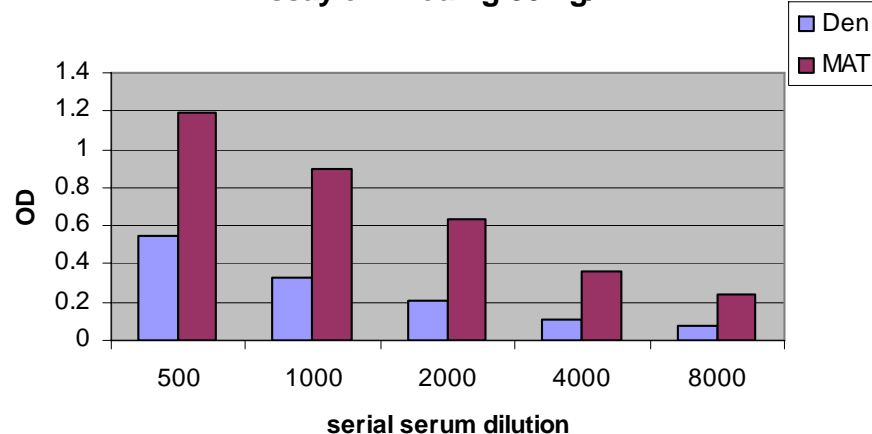
rLipL21 = 15 ng/ml

rLipL41 = 30 ng/ml

rLipL32 = 30 ng/ml

rLoa22 = 30 ng/ml

### Assay on rLoa Ag 30 ng/ml



Serum dilution 1:1000 was selected.

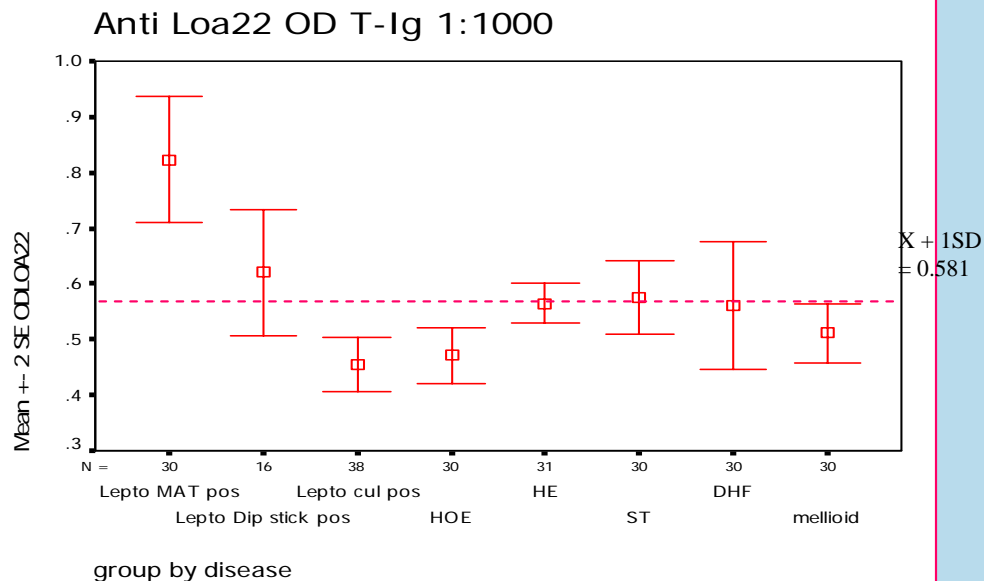
Conjugation anti Total Ig or anti IgM with HRP of 1:4000 dilution was used.

Utilized the ABTS substrate





## OD profile of anti Loa22



Dependent Variable:  
ODLOA22  
Games-Howell

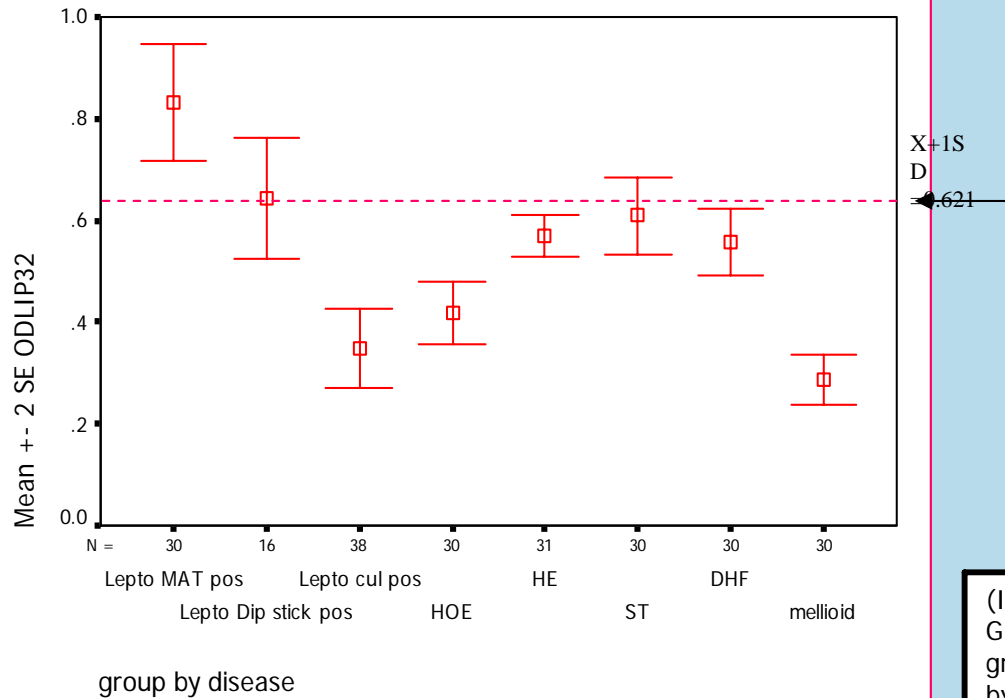
**There were significant difference between**

Lepto MAT pos  
VS  
Lepto cul pos  
HOE  
HE  
ST  
DHF  
melioid

(I) GROUP group by disease	(J) GROUP group by disease	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1 Lepto MAT pos	2 Lepto Dip stick pos	.20360	.080017	.207	-.05231	.45950
	3 Lepto cul pos	.36824(*)	.061783	.000	.17072	.56577
	4 HOE	.35227(*)	.062099	.000	.15382	.55071
	5 HE	.25821(*)	.059408	.003	.06677	.44965
	6 ST	.24793(*)	.065316	.009	.04064	.45523
	7 DHF	.26133(*)	.080625	.039	.00783	.51483
	8 melioid	.31190(*)	.062594	.000	.11213	.51167



# Anti LipL32 OD T-Ig 1:1000



# OD profile of anti LipL32

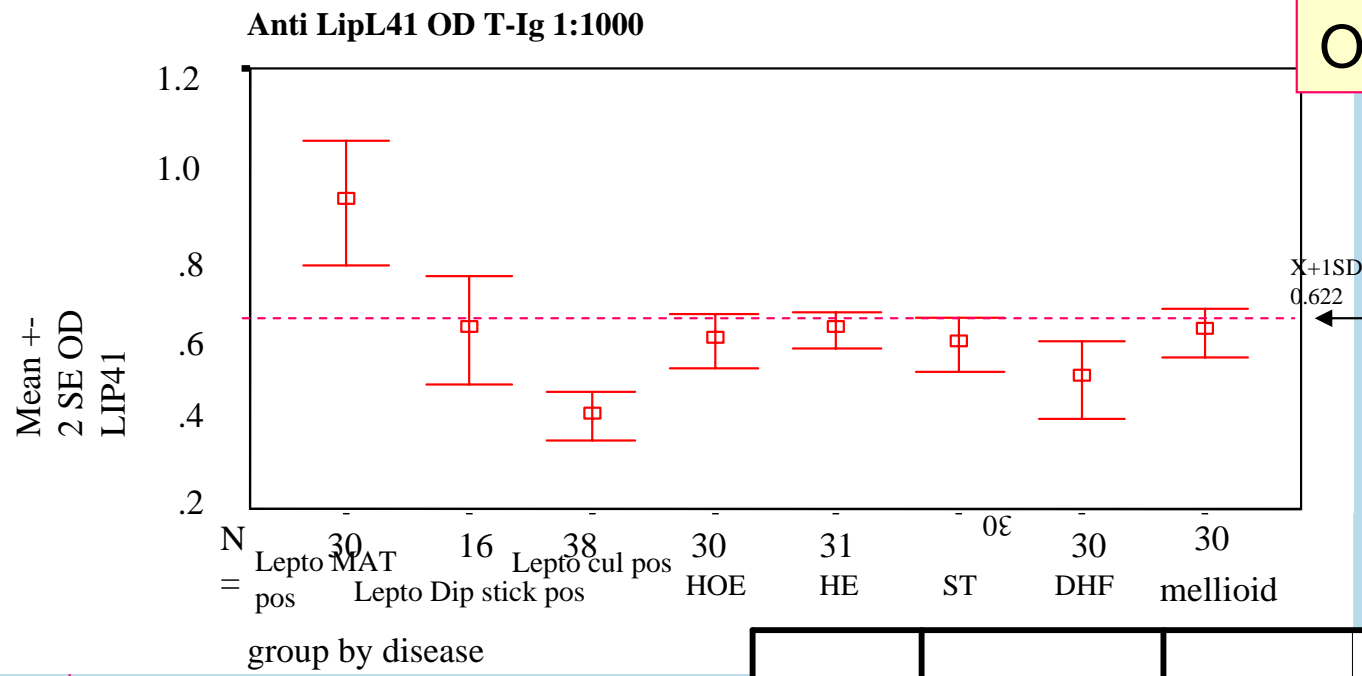
Dependent Variable: ODLIP32  
Games-Howell

**There were significant difference between**

- Lepto MAT pos
- VS
- Lepto cul pos
- HOE
- HE
- ST
- DHF
- melliod

(I) GROUP group by disease	(J) GROUP group by disease	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1 Lepto MAT pos	2 Lepto Dip stick pos	.18822	.082969	.336	-.07748	.45391
	3 Lepto cul pos	.48323(*)	.069747	.000	.26320	.70326
	4 HOE	.41407(*)	.065624	.000	.20548	.62265
	5 HE	.26310(*)	.060968	.003	.06698	.45921
	6 ST	.22373(*)	.069080	.041	.00530	.44217
	7 DHF	.27670(*)	.066385	.003	.06598	.48742
	8 melliod	.54703(*)	.062786	.000	.34618	.74789

# OD profile of anti LipL41



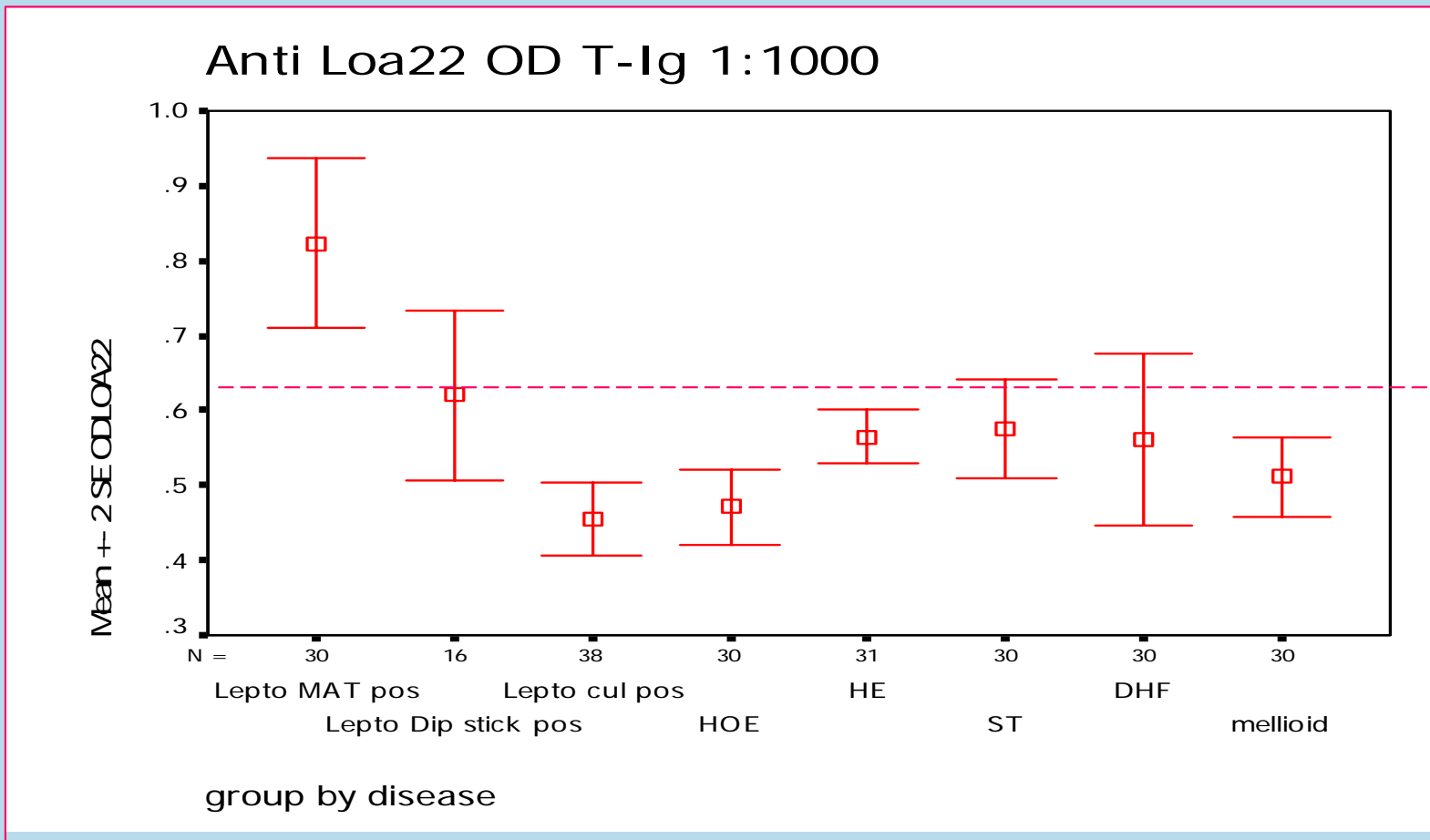
Dependent Variable: ODLIP32  
Games-Howell

**There were significant difference between**

Lepto MAT pos  
VS  
Lepto cul pos  
HOE  
HE  
ST  
DHF  
melloid

(I) GROUP group by disease	(J) GROUP group by disease	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1 Lepto MAT pos	2 Lepto Dip stick pos	.28907	.093639	.064	-.00930	.58745
	3 Lepto cul pos	.48602(*)	.075792	.000	.24291	.72912
	4 HOE	.31620(*)	.077003	.004	.06985	.56255
	5 HE	.29065(*)	.073505	.008	.05330	.52800
	6 ST	.32300(*)	.077442	.004	.07548	.57052
	7 DHF	.40197(*)	.083348	.000	.13803	.66590
	8 melloid	.29617(*)	.075994	.008	.05247	.53986

# Setting up the cut off value as differential diagnosis



X + 2 SD  
= 0.625

## Mean ± SD of anti Loa22

Lepto MAT pos = 0.823 ± 0.310

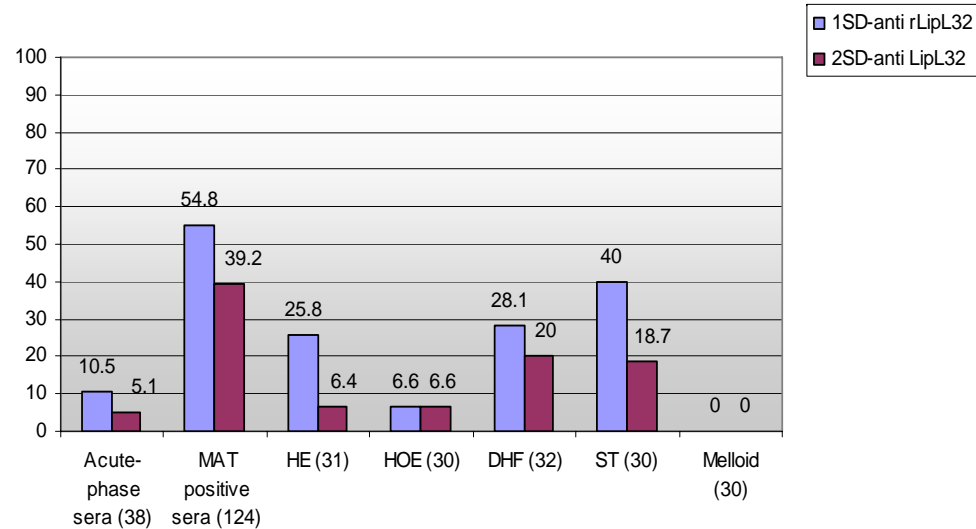
Control group = 0.537 ± 0.044 (HOE, HE, ST, DHF, Melioid)

Cut off value = mean + 1 SD = 0.537 + 0.044 = 0.581

mean + 2 SD = 0.537 + 0.088 = 0.625

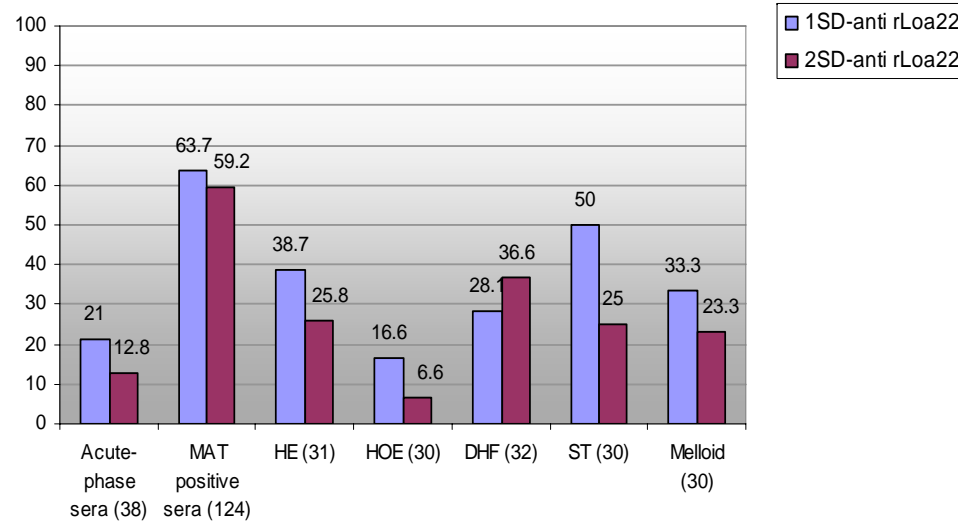


### Percent positive of anti rLipL32



Number of positive case (%) based on the cut off value derived from **1SD** and **2SD** among each studied group was determined;

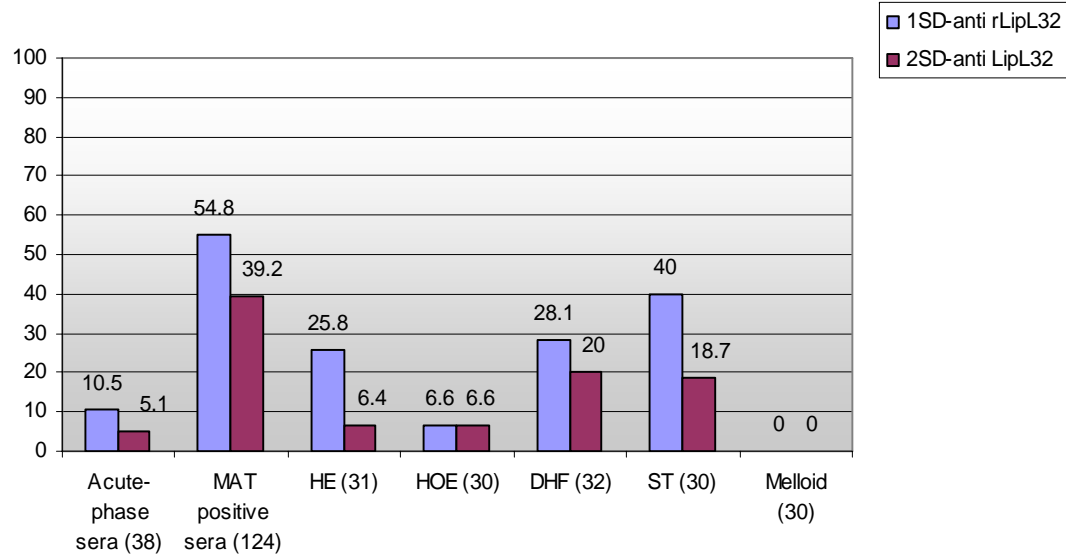
### Percent positive anti-rLoa22



LipL32 was able to differentiate melioidosis patient and normal human in non-endemic area, from Leptospirosis patient well.

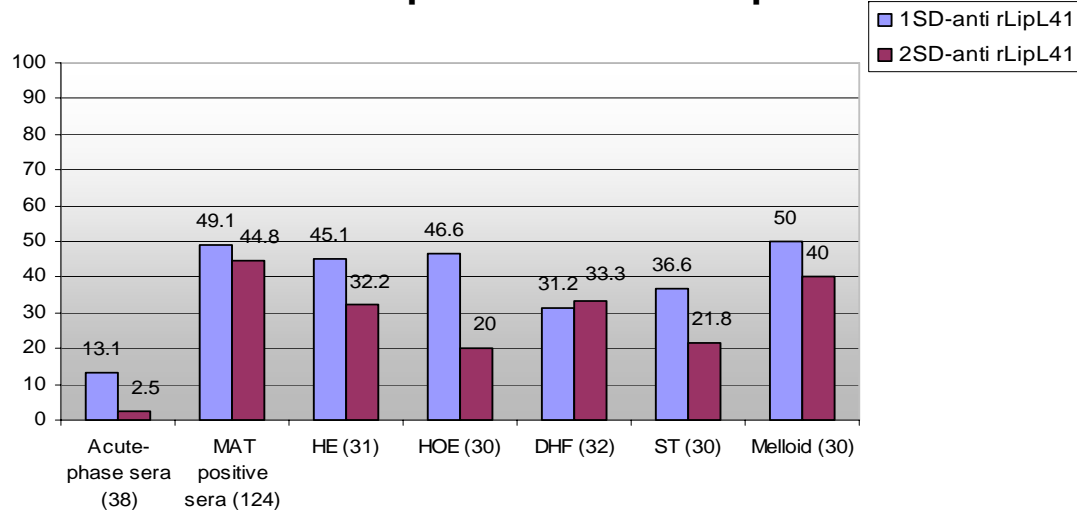


### Percent positive of anti rLipL32



Number of positive case (%) based on the cut off value derived from **1SD** and **2SD** among each studied group was determined;

### Percent positive of anti rLipL41



## Efficacy of ELISA-based recombinant protein (MAT as gold standard)

Percentage	rLoa22	rLipL32	rLipL41
Sensitivity	76.60	56.60	70.0
Specificity	76.58	88.70	74.6
Accuracy	76.59	84.60	74.0



# Comparative to previous study

- ◆ Whole-cell *Leptospira*-based serologic assays using enzyme-linked immunosorbent assay (ELISA) demonstrated the sensitivities and specificities of these tests ranged from 28 to 72% and 10 to 99%, respectively (McBride et al. ,2007).
- ◆ The major limitation of whole-cell *Leptospira*-based serologic assays is the low sensitivity (<67%) to samples obtained from patients in the first week of illness.
- ◆ Low sensitivity was revealed among residence of endemic area.



## Efficacy of ELISA-based recombinant protein to detect suspected Leptospirosis cases

The cut off value derived from mean + 2 SD of OD of control group

Group by disease	Total Number	Positive	anti rLip41	Positive	anti rLipL32	Positive	anti rLoa22
	of Cases	OD $\geq 0.666$	% positive	OD $\geq 0.754$	% positive	OD $\geq 0.625$	% positive
1 MAT pos	125	56	44.8	49	39.2	74	59.2
2 Dip stick pos	16	6	37.5	5	31.2	7	43.7
3 Lepto cul pos	39	1	2.5	2	5.1	5	12.8
4 West ern pos	29	12	41.3	16	55.1	14	48.2
Total	209	75	35.8	72	34.4	100	47.8

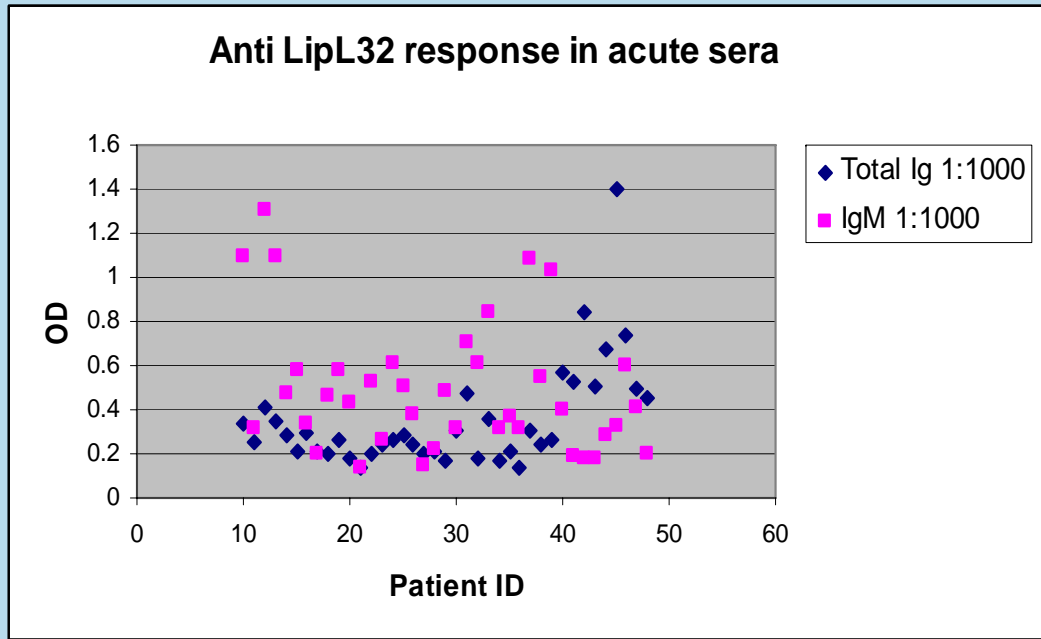




**IgM-based ELISA was  
proposed to use to indicate  
acute infection.**



# OD of anti LipL32 (IgM) 1:1000



When cut off was set as;  
 $OD \geq X + 1SD = 0.662$

Positive 7 out of 39 (17.9%)

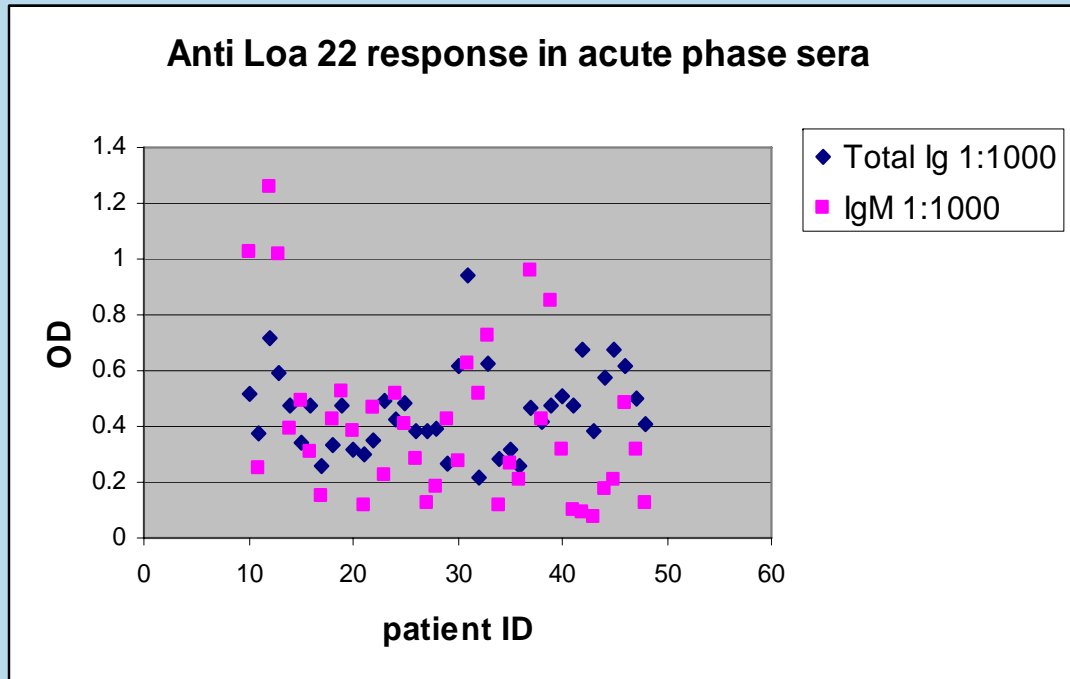
IgM 1000 LipL32			
group by disease	Mean	N	Std. Deviation
Lepto MAT pos	0.52367	30	0.273459
Lepto cul pos	0.48808	39	0.296085
HOE	0.51088	24	0.245864
HE	0.65963	30	0.333736
ST	0.576	30	0.280228
DHF	0.55463	30	0.264361
meliod	0.26437	30	0.164975
Total	0.51007	213	0.290658

Cut off value was derived from control group

$$X + 1SD = 0.513 + 0.149 = 0.662$$



# OD of anti Loa22 (IgM) 1:1000



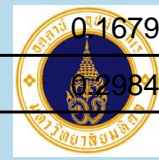
When cut off was set as;  
 $OD \geq X + 1SD = 0.650$



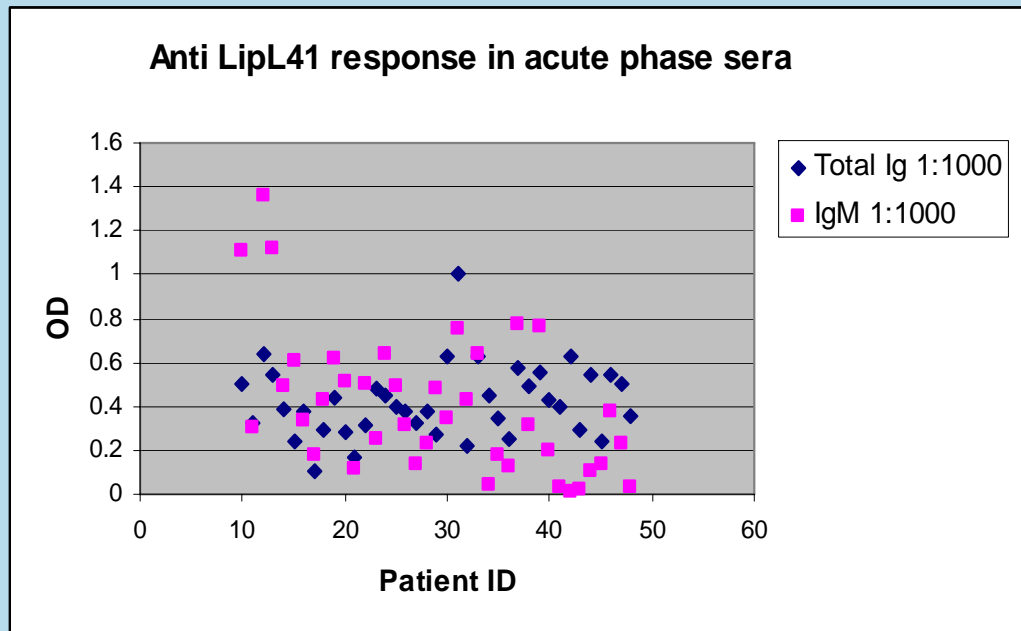
Positive 5 out of 39 = 12.8%

IgM 1:1000 Loa22			
group by disease	Mean	N	Std. Deviation
Lepto MAT pos	0.4288	30	0.271103
Lepto cul pos	0.40638	39	0.290303
HOE	0.53733	24	0.262881
HE	0.68613	30	0.343947
ST	0.4984	30	0.271616
DHF	0.44793	30	0.243178
meliod	0.20233	30	0.167954
Total	0.45377	213	0.298404

$X + 1SD = 0.474 + 0.176$   
 $= 0.650$



# OD of anti LipL41 (IgM) 1:1000



When cut off was set as;  
 $OD \geq X + 1SD = 0.649$



Positive 6 out of 39 (15.3%)

IgM1:1000 LipL41			
group by disease	Mean	N	Std. Deviation
Lepto MAT pos	0.2887	30	0.225791
Lepto cul pos	0.40272	39	0.322333
HOE	0.54221	24	0.267474
HE	0.7145	30	0.363705
ST	0.3735	30	0.282647
DHF	0.38283	30	0.2962
meliod	0.10703	30	0.133968
Total	0.39773	213	0.327354

$$X + 1SD = 0.424 + 0.225 = 0.649$$



## IgM positivity among studied group

N group by disease (N)	IgM 1:1000		
	Anti LipL32	Anti loa22	Anti LipL41
Lepto MAT pos (30)	9	7	2
Lepto cul pos (39)	7	6	6
HOE (24)	4	6	6
HE (30)	15	15	16
ST (30)	11	8	5
DHF (30)	7	5	5
Melioid (30)	1	0	0
Total (213)	54	47	40

Interpretation of healthy individuals of endemic area was concerned, as these people may be exposed to antigen recently, with no infection.

Patient's symptom should also be considered.

Co-infection of Leptospirosis with ST and DHF, could elevate Ab to *Leptospira*.



As IgM antibodies become detectable during 5 – 7 days after the onset of symptoms



The low percentage of detection of the acute sera, indicate the less amount of IgM at that point.



The second sera was thus required, as at first time point of sera collection, IgM was not raised up.



## Relation of MAT titer to ELISA assay

(MATtiter) total cases	AntiLipL32 Positive (%)	AntiLipL41 Positive (%)	Anti Loa22 Positive (%)
(100) 54	25 (46)	29 (53)	38 (70)
(200-800) 64	22 (34)	24 (37)	30 (46)
(≥1000) 20	7 (35)	9 (45)	14 (70)

The MAT cut off titer at 1:100 as positive, was in question?



# In summary

- ◆ rLipL32 gave the most accurate result (84.6%) in discrimination among other febrile illness.
- ◆ rLoa22 gave the moderately accurate (76%), while the sensitivity was higher (76%) than rLipL32 (56.6%)
- ◆ ELISA assay was able to indicate the positivity among MAT negative samples, that gave positive results by Lepto Dipstick and Western blot.





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