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THE PATENTS ACT, 1970

(39 of 1970)

COMPLETE SPECIFICATION

(See section 10 and rule 13)

1**. TITLE OF THE INVENTION** : **"**B and T cell specific peptides of l*eptospiral protein LK90* for the diagnosis leptospirosis*"*	
2. APPLICANT(S) **(a) NAME: Bharathidasan University** **(b) NATIONALITY: INDIAN** **(c) ADDRESS: Palkalai Perur Tiruchirappalli – 620024 Tamil Nadu**	
3. PREAMBLE TO THE DESCRITION	
PROVISIONAL SPECIFICATION	**COMPLETE**
4. DESCRIPTION :Attached	
5. CLAIMS : Attached	
7. ABSTRACT OF THE INVENTION : Sheet Attached	

TITLE OF THE INVENTION

"B and T cell specific peptides of l*eptospiral protein LK90* for the diagnosis leptospirosis"

RELATED APPLICATION

This application is a continuation of application Number **3950/CHE/2014** filed with the Indian Patent office

FIELD OF THE INVENTION

The present invention relates to a B and T cell specific peptides, more specifically to a *leptospiral* protein LK90. The invention further relates to a method of diagnosis of leptospirosis B and T cell specific peptides of l*eptospiral protein LK90* for the diagnosis leptospirosis.

OBJECTIVE OF THE INVENTION

It is an objective of the present invention, to provide B and T cell specific peptides of l*eptospiral protein LK90* for the diagnosis leptospirosis

It is another objective of the present invention to provide a method of diagnosis of leptospirosis via B and T cell specific peptides of l*eptospiral protein LK90* for the diagnosis leptospirosis

RELEVANT PRIOR ART OF THE INVENTION

Leptospirosis also known as field fever,rat catcher's yellows, and pretibial fever is an important public health problem both in industrialized and developing countries**.** It is caused by spirochaetes of the genus *Leptospira*. Clinical manifestation of acute leptospirosis ranges from a mild febrile illness to more severe icteric Weil's disease. Symptoms can range from none to mild such as headaches, muscle pains, and fevers to severe with bleeding from the lungs or meningitis. If the infection causes the person to turn yellow, have kidney failure and bleeding it is then known as Weil's disease If it causes lots of bleeding from the lungs it is known as severe pulmonary haemorrhage syndrome

There are ten different types of *Leptospira* that cause disease in humans. It is transmitted by both wild and domestic animals. The most common animals that spread the disease are rodents It is often transmitted by animal urine or water containing animal urine coming into contact with breaks in the skin, the eyes, mouth, nose or vagina. In the developing world the disease most commonly occurs in farmers and poor people who live in cities. In the developed world it most commonly occurs in those involved in outdoor activities in warm and wet areas of the world Diagnosis is by growing the bacteria from a blood sample, finding its DNA in the blood, or looking for antibodies against the infection.[1]

Leptospirosis is under-diagnosed for many reasons, including difficulty in distinguishing clinical signs from those of other endemic diseases and a lack of appropriate diagnostic laboratory services. Misdiagnosis of leptospirosis leads to severe complications like kidney damage, liver failure, respiratory distress, and meningitis and also leads to death. The gold standard technique for the diagnosis of leptospirosis is the Microscopic Agglutination Test (MAT). MAT requires experts for interpretation of the results and paired sera to achieve sufficient sensitivity. The sensitivity of other rapid and less complicated serological techniques such as Lepto-dipstick, Lepto-dridot, immunoflourescence assays (IFA) is inappreciable especially during early phase of the disease.

Several attempts to standardize the serological tests for leptospirosis based on the ELISA have been made, but considerable variations in sensitivity and specificity have been obtained. In this regard, an alternative method for the diagnosis of acute leptospirosis is required. To meet out the need peptide based ELISA can be widely used for sero-diagnosis of leptospirosis. The highly conserved peptides of immunogenic proteins provide the advantage of enhanced specificity and can be easily implemented into a simple, rapid, sensitive and relatively cheap diagnostic kit.

In view of the foregoing, there is a need for peptides and methods that provide enhanced diagnosis of leptospirosis in the early stages and prevent spreading of disease.

DETAILED DESCRIPTION OF THE INVENTION

B-cell epitopes for leptospiral protein LK90 was predicted using BCPred (ailab.cs.iastate.edu/bcpreds/ - 6k; EI-Manznlzwy *et al*., 2008; Chen *et al*., 2007). B-cell epitopes having BCPred score >0.9 and VaxiJEN score >0.4 were selected for the prediction of T- cell epitopes using MHCPred analysis (http://www.jenner.ac.uk/MHCPred; Guan *et al*., 2003). MHCPred implements the statistical models for both Class I alleles (HLA-A*0101, HLA-A*0201, HLA-A*0202, HLA-A*0203, HLA-A*0206, HLA-A*0301, HLA-A*1101, HLA-A*3301, HLA-A*6801, HLA-A*6802 and HLA-A*3501), ClassII alles (HLA-DRB*0101, HLA-DRB*0401, HLA-DRB*0701, HLA-I*Ab, HLA-I*Ad, HLA-I*Ak, HLA-I*Eg, HLA-I*Ek, HLA-I*As, HLA-I*Ed) and others (TAP) (Guan *et al*., 2006). The results of computational analysis included peptides and their corresponding IC50 value, which implies the binding affinity. Peptides with predicted binding affinities <500nM were considered as good binders.

Three peptides “HSSNNSVATC”, “SNAQKNQGNC” and “DHHTQSSYTC” with greater BCP red and VaxiJen score were selected for chemical synthesis. Further these epitopes were found to be highly conserved among all pathogenic *Leptospira* species by multiple sequence alignment. The three peptides were synthesized with biotin at the N-terminal linked to the peptide sequence through a spacer sequence of “SGSG” to improve the solubility of the peptide and the ease to perform ELISA.

Antibodies to these epitopes were readily detected in human sera in the format of epitope-blocking ELISA as described previously (Timoney *et al.,* 2010). The sensitivity and specificity of IgM ELISA using peptides in ELISA format was in the range of 99.2 – 100%. Among all the peptides used in ELISA, peptide 3 had 100% sensitivity and specificity followed by peptide 1 and peptide 2. The cross-reactivity of the peptides (for diseases other than leptospirosis) were found to be in the range of 1.7 – 3.9%.

The cut off value (Mean + 2SD) for peptides were 0.190, 0.191 and 0.194 for peptide1; 2; 1+2 respectively. The sensitivity and specificity of IgM ELISA was in the range of 97 – 98%.

The sensitivity and specificity of peptide-based ELISA for rLig-C did not differ significantly. Among all the peptides used in ELISA, peptide 2 has 97.9% sensitivity followed by peptide 1. When peptides are used in combinations, peptide 1 and 2 in combination gave an increased sensitivity 97.9%. The cross-reactivity of the peptides was found to be in the range of 2.3 – 4.1% among the sera from diseases confirmed other than leptospirosis.

Thus the B and T-cell specific peptide based ELISA showed a sensitivity of 100% which is ~13% and ~6% higher than that of the whole cell lysate and the corresponding recombinant proteins specific ELISA respectively substantiating the peptides as promising diagnostic candidates. Thus, immunogenic peptide based-ELISA should be considered as MAT alternatives in primary and secondary health care centres, not only based on their simplicity and rapidity but also on the affordability for the people in developing countries like India where leptospirosis has been established as an endemic disease.

EXAMPLE

The present example is only a method of performing this invention and in no way construing to act as a limitation of the scope of the invention

Case patients and control subjects

A total of 596 sera samples (Table 1) from the serum bank of Medical Microbiology Laboratory, Department of Microbiology, Bharathidasan University were used for the present study. Of the 596 sera samples, 123 had a laboratory confirmed diagnosis of leptospirosis (isolation of leptospires from blood or urine, seroconversion: negative to a titre of 1:160 or more or four-fold rise in titre, IgM-ELISA with a titre of ≥1:160 using heat extracted antigen), 70 were laboratory unconfirmed cases, 135 were seronegative healthy controls, and 267 subsequently diagnosed as having other illness based on laboratory and radiological evidence. Of the 267 cases, 98 had typhoid, 39 had malaria, 53 had hepatitis and 77 had dengue. Informed written consent was obtained from both cases and controls before blood sampling, and the study protocol was approved by the Institutional Ethics Committee (IEC) of Bharathidasan University (DM/2007/101/373/ Project No.2) as well as permitted by

the Directorate of Health Services (Ref. No. 5796/ TV 1/07), Tamilnadu. The obtained sera samples were stored at -80°C until use.

Table 1: Case definition and grouping of the patients included in the present study

Groups	Cases	Number
A	Clinically suspected laboratory confirmed Leptospirosis[†]	123
B	Clinically suspected laboratory negative Leptospirosis[‡]	70
C	Seronegative healthy controls	135
D	Hepatitis	53
E	Typhoid	98
F	Dengue	77
G	Malaria	39

[†]Confirmed cases for Leptospirosis; [‡]Clinically suspected but serologically negative

Leptospira culture and microscopic agglutination test (MAT)

A panel of 12 reference strains were used for MAT which included the following serogroups: Australis (serovarAustralis, strain Ballico), Autumnalis (serovarAutumnalis, strain Akiyami A), Ballum (serovarBallum, strain Mus 127), Bataviae (serovarBataviae, strain Swart), Canicola (serovarCanicola, strain Hond Utrecht IV), Icterohaemorrhagiae (serovarIcterohaemorrhagiae, strain RGA), Grippotyphosa (serovarGrippotyphosa, strain Moskva V), Hebdomadis (serovarHebdomadis, strain Hebdomadis), Javanica (serovar Poi, strain Poi), Pomona (serovar Pomona, strain Pomona), Sejroe (serovarHardjo, strain Hardjoprajitno) and Pyrogenes (serovarPyrogenes, strain Salinem). Apart from the reference strains non-pathogenic strain Patoc I of serovarSemaranga were also maintained. All leptospiral reference strains were maintained in EMJH medium at Medical Microbiology Laboratory, Bharathidasan University, Tiruchirappalli. The MAT was performed using 7-day old cultures grown at 30ºC in EMJH medium (Faine*et al*., 1999).

Peptide based Enzyme Linked Immunosorbent Assay (ELISA)

Checkerboard titrations were performed to determine the optimal concentrations of leptospiral peptides. The wells of 96 well flat-bottom plates (NuncMaxiSorp®) were coated

with streptavidin (Sigma-Aldrich, St. Louis, MO; 0.5 mg in 100 mL water), washed and blocked with 4% non-fat dry milk. The peptides at a concentration of 0.2 µg/well were added to streptavidin coated plates using carbonate coating buffer (pH 9.6), and incubated overnight at 4°C. The peptide coated wells were blocked with 4% non-fat dry milk. Serum samples (1:200) in triplicates were added and incubated for 1 h at 37°C. Bound IgM was detected using HRP-conjugated rabbit anti-human IgM (Sigma-Aldrich, St. Louis, MO) at a dilution of 1:8,000. Plates were developed with *o*–phenylenediamine (Sigma-Aldrich, St. Louis, MO). The reaction was stopped with the addition of 50 µl of 1 N H_2SO_4, and the optical density was measured at 490 nm using a ELISA reader (Bio-Rad, USA).

Statistical Analysis

Data were analyzed and plotted using SigmaPlot 11.0 or graph pad prism version 5.0 software. Cut-off values for each diagnostics were defined as the corresponding Mean+2SD calculated from the sera of normal healthy controls. Sensitivity was defined as the percentage of the laboratory-confirmed cases of leptospirosis whose serum samples gave mean OD greater than the relevant cutoff value. Specificity was calculated as the percentage of the control individuals whose samples gave mean OD below the relevant cut-off value. The positive and negative predictive values (PPV and NPV respectively) are the proportions of true positive and true negative results. All statistical values were determined using Epi Info version 6.0 (Centers for Disease Control and Prevention, Atlanta, GA).

Results

The sensitivity and specificity of IgM ELISA using peptides in ELISA format was in the range of 99.2 – 100%. Among all the peptides used in ELISA, peptide 3 had 100% sensitivity and specificity followed by peptide 1 and peptide 2. The cross-reactivity of the peptides (for diseases other than leptospirosis) was found to be in the range of 1.7 – 3.9%.

The cut off value (Mean + 2SD) for peptides were 0.258, 0.248, 0.279, 0.266, 0.293, 0.305, 0.292 for peptide1; 2; 3; 1+2+3; 1+2; 1+3; 2+3 respectively (Figure 1). The sensitivity and specificity of IgM ELISA on comparison with MAT is given in Table 2.All the peptides have good sensitivity and specificity. Among all the peptides used in ELISA, peptide 3 has 100% sensitivity and specificity followed by peptide 1 and peptide 2. When peptides are used in

combinations, peptide 1, 2 and 3 in combination gave an increased sensitivity and specificity of 100% followed by peptide 2 and 3, peptide 1 and 3, peptide 1 and 2.

Table 2: Sensitivity, specificity, PPV and NPV among different group of leptospirosis patients using predicted peptides of LK90 in IgM-ELISA

Peptides of LK90	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Peptide 1	99.2	100	100	98.6
Peptide 2	97.6	92.9	96	95.6
Peptide 3	100	100	100	100
Peptide 1,2, 3	100	92.9	96.1	100
Peptide 1 and 2	95.9	84.3	91.5	92.2
Peptide 1 and 3	97.6	100	100	95.9
Peptide 2 and 3	99.2	97.1	98.4	98.6

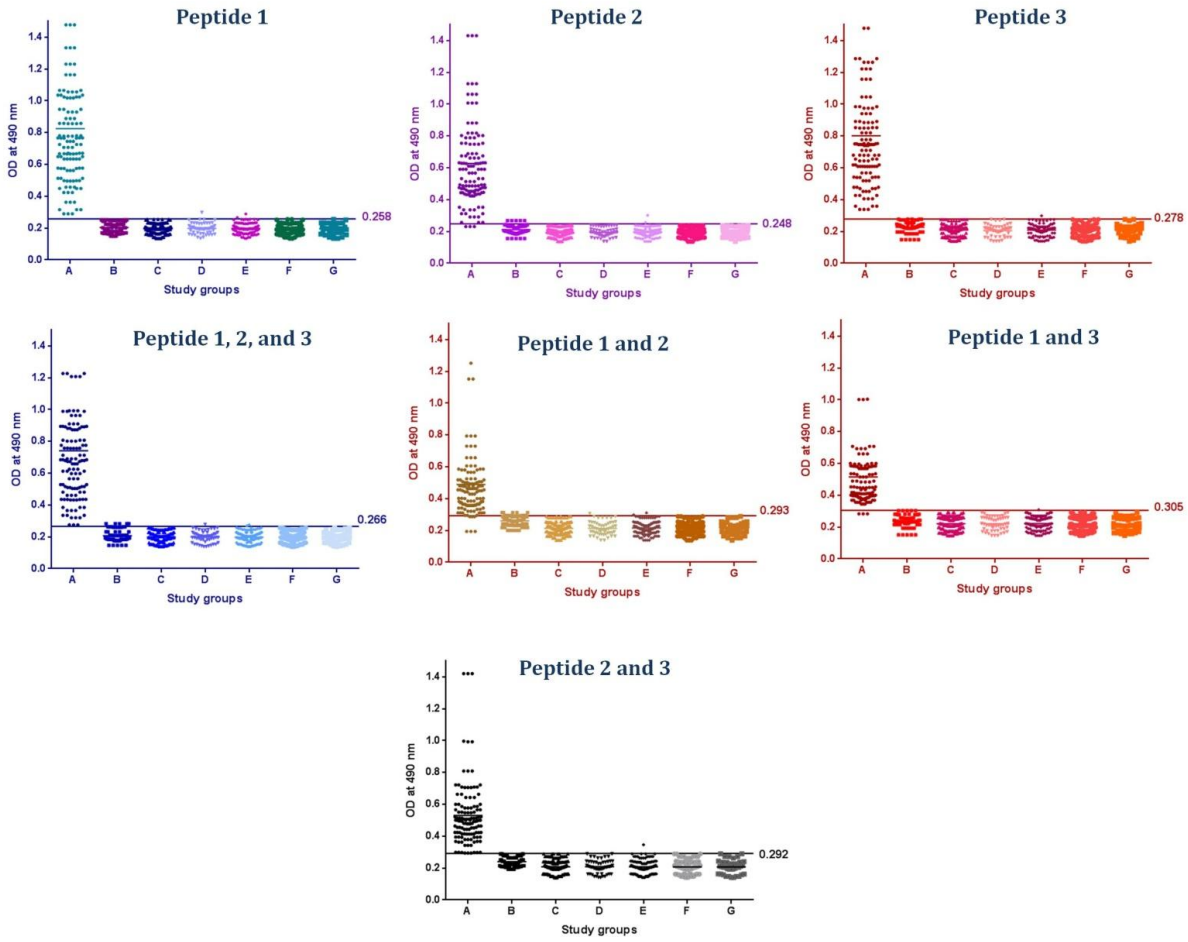

Figure 1: Evaluation of IgM-ELISA with immunogenic peptides of LK90 against sera from patients' with different clinical manifestations: Study groups are indicatedon the *x* axis and the optical density (OD) at 490 nm on the *y* axis. Study groups were as described in Table 1. The solid reference line represents the cut-off values for each antigens with the absolute cut-off values on the right.

Claims

1. Pathogenic *Leptospira* Peptide sequence HSSNNSVATC", "SNAQKNQGNC" and "DHHTQSSYTC" for identification of diagnosis of leptospirosis infection
2. A method for diagnosis of sample infected with leptospirosis, wherein the method comprises identification of complementary binding to the sequences ", "SNAQKNQGNC" and/or "DHHTQSSYTC"
3. A method according to claim 2, for diagnosis of leptospirosis infection, where in the method comprises performing ELISA based diagnosis

ABSTRACT OF THE INVENTION

The present invention pertains to three peptides “HSSNNSVATC”, “SNAQKNQGNC” and “DHHTQSSYTC” with greater BCP red and VaxiJen score were selected for chemical synthesis. Further these epitopes were found to be highly conserved among all pathogenic *Leptospira* species by multiple sequence alignment. The three peptides were synthesized with biotin at the N-terminal linked to the peptide sequence through a spacer sequence of “SGSG” to improve the solubility of the peptide and the ease to perform ELISA.