Available online at **www.ijmrhs.com**

ISSN No: 2319-5886

International Journal of Medical Research & Health Sciences, 2016, 5, 4:72-77

Investigation of protein in blood serum of dengue patients by SDS-PAGE technique

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ABSTRACT

Dengue viruses belong to the genus flavivirus within the Flaviviridae family. It is self limited viral infection that is transmitted between people by mosquitoes. The dengue virus genome encodes three structural and seven nonstructural proteins. Dengue virus is categorized into four serotypes,so far it is the major health problem and 2.5 billion people are at danger of this disease among them about 975 million of them live in urban areas in tropical areas of Southeast Asia. The sample of dengue positive patients were collected and Serum protein of dengue and healthy individuals can be analyzed and exploited by using a technique sodium dodecyl sulphate Polyacrylamide gel electrophoresis .For this purpose serum of healthy and infected patients were taken and infection in individuals were confirmed by using ELISA IgG, IgM and NS1 antigen test. From the SDS –PAGE analysis a protein profile with molecular weight ranging from 200kda to 10kda were analyzed .One serum protein marker that has a molecular weight of about 48kda that is specifically present in dengue patients but not in the healthy person . The recent research concluded that method adopted for protein analysis is sensitive and reliable. Careful and consistent investigation is required in the future to identify these serum protein marker and functions of these protein in the dengue disease.

Keywords: Dengue patients, sodium dodecyl sulphate Polyacrylamide gel electrophoresis,, ELISA, Serum protein marker.

INTRODUCTION

Dengue fever is a mosquito borne viral disease in human beings. Worldwide an approximately 2.5 billion people are at danger of disease, about 975 million of them live in Tropical and Subtropical regions of the world like Sri Lanka, Indonesia, Philippines, Thailand, India and Pakistan etc. In Pakistan the first case of dengue fever was reported in 1994 from Karachi, since then it has become an epidemic in Lahore while a few cases have been identified in other parts of the country [1]. Four different types of dengue virus are responsible for dengue fever .One or more than one type can enter into female Aedes mosquito while biting an infected person and when these mosquitoes bites other human inject viruses along with saliva. Recovery after infection with one type of virus can produce lifelong Immunity to like that type but severe complication can occur on secondary infection by other serotype of virus[2]. The structure of the virus is roughly spherical with a diameter of about 50 nm. The core of the virus is the nucleocapsid structure that is composed of the viral genome accompanied with C protein. The nucleocapsid is covered by a membrane called the viral envelop, a lipid bilayer that is taken from the host and fixed in the viral envelope are 180 sets of the E and M protein that seep through the lipid bilayer .These protein form a defensive outer layer that blocks the entry of the virus into a human cells . The dengue virus genome is a positive sense single strand of RNA .The viral genome is replaced as a single, long polypeptide and then cut into ten proteins

.The dengue virus genome encodes three structural (Capsid ,Membrane and Envelop and seven non structural (NS1,NS2A,NS2B,NS3,NS4A,NS4B and NS5) proteins. These nonstructural proteins play a vital roles in viral replication [3]. In regions where dengue is local the sickness is often clinically non specific notably in children with symptoms of a viral syndrome that has a collection of local names. Important risk element affecting ratio of victims who has severe infections during epidemic transference include the strains and serotype of the infecting virus and immune status, age and genetic history of the human host[4]. Laboratory investigation of dengue is associated directly by identification of viral components in serum or indirectly by serological tests[5].During the febrile phase confirmation of viral nucleic acid in the serum by test of reverse transcriptase polymerase chain reaction (RT-PCR) assay or identification of the virus expressed soluble nonstructural protein 1 (NS1) by tests of enzyme linked immunosorbant assay (ELISA) or the lateral flow rapid test, are enough for a confirmatory diagnosis. For primary infection the diagnostic sensitivity of NS1 present in the febrile phase can exceed 90% and is lower in secondary infection[6] .Serological tests of dengue depends on the presence of high ratio of serum IgM that bind dengue virus antigen in an (ELISA) or a lateral flow rapid test .IgM can be identified as early as 4 days after the onset of fever .Inition victims with secondary disease mount rapid anamnestic antibody responses in which dengue virus reactive IgG may preferred over IgM[7]. New vector control struggles include the production of genetically modified male mosquitoes that sterilize the wild type female population and decreasing egg output and the population size of the next generation that would be available for potential transmission of the dengue virus. The leading dengue vaccine candidate Chimerivax (sanofi pasture)is a tetravalent formulation of attenuated yellow fever 17D vaccine strain showing the dengue virus prM and E proteins .It has been difficult to synthesize a vaccine for dengue that is safe and elicit balanced neutralizing antibody responses to all 4 serotypes[8].

MATERIALS AND METHODS

Sample collection and Serum preparation: Blood samples of dengue positive patients were collected from holy family hospital Rawalpindi. In which they provide us patient's personal information, they also give us patient's lab registration number all the samples were in the form of blood. In order to separate the serum from blood samples were subjected to centrifugation at 10000rpm for 1 minute. First of all took the desired number of coated strips into the holder. Calibrator and positive negative control are ready to use .prepare 1:21 dilution of the patient's samples by adding 10ul of serum sample and 200ul of serum diluents. Then add 100ul of the diluted serum, positive and negative control into the antigen containing wells and then tap the holder to remove air bubbles from the liquid and mix well and then incubated for 20 minutes at room temperature .Then removed all the liquid from the well and wash the well with 1X buffer three times and then blot on paper towel .and then poured 100ul of enzyme conjugate complex to each washed well and again incubated at room temperature for 20 minutes then removed all the excess enzyme conjugate from the wells and then wash well with the washing buffer blot on absorbance paper. Then added 100ul of Tetramethylenebenzidine substrate in all the wells and incubated again at room temperature for 10 minutes and add 100ul of stop solution read optical density at 450nm using Elisa reader within 20 minutes. For the preparation of Protocol of immunoglobulin M .First of all took the desired number of coated strips into the holder. Calibrator and positive negative control are ready to use .prepare 1:21 dilution of the patient's samples by adding 10ul of serum sample and 200ul of serum diluents. Then add 100ul of the diluted serum, positive and negative control into the antigen containing wells and then tap the holder to remove air bubbles from the liquid and mix well and then incubated for 20 minutes at room temperature .Then removed all the liquid from the well and wash the well with 1X buffer three times and then blot on paper towel .and then poured 100ul of enzyme conjugate complex to each washed well and again incubated at room temperature for 20 minutes then removed all the exess enzyme conjugate from the wells and then wash well with the washing buffer blot on absorbance paper. Then added 100ul of Tetramethylenebenzidine substrate in all the wells and incubated again at room temperature for 10 minutes and add 100ul of stop solution read optical density at 450nm using Elisa reader within 20 minutes.

Procedure for the nonstructural 1 protein detection

First of all taken the blood or serum of suspected dengue patients and then add 100ul blood with the help of micropipette on the sample insertion hole on the device and leave on it for fifteen minutes .Then read the result on the test region and control region. If only one colored line appear in the control region then the test is negative. If two colored bands appear one in the control region and one in the test region then the test is positive.

Table:1.Preparation of 12% SDS –PAGE

Resolving gel

dH_20	1.6ml
30% acrylamide mix	2 ml
1.5M Tris pH8.8	1.3ml
10% SDS	50µl
10% ammonium per sulfate	50µl
TEMED	4µl

Stacking gel

dH_20	680µl
30% acrylamide mix	170µl
1.0M Tris pH 6.8	130µl
10% SDS	10µl
10% ammonium per sulfate	10µl
TEMED	3µl

After dialysis and concentrated the solution it was run on SDS-PAGE to determine the presence of the desired protein by determining its molecular weight. First of all the gel was polymerized. A spacer plate and short plate were adjusted in a clamp and set on the stand of BIO-RADSDS apparatus. Then the resolving gel was poured in between the plate's .After pouring the gel solution ethanol or propanol was added on the resolving gel to stop oxidation of the gel by air. It was kept for 30 minutes for polymerization. After the solidification of gel excess ethanol was removed and poured the stacking gel between the plates upon the solid resolving gel then it was allowed to solidify at room temperature. Meanwhile sample was prepared by taking 10ul of the serum and 90ul of 20mM Tris buffer and then out of this solution 50ul of the serum was taken in an eppondrof tube and add 10ul of the loading dye and then boiled for 3-5 minutes and allow cooling. After the solidification of stacking gel inserted the comb in the gel for the formation of well and set the plate in the tank containing running buffer i.e. tris –glycine buffer after setting the plate removed the comb and the performed samples was loaded in the wells along with the protein ladder which give the standard weight of protein. Then the gel was run between 80-100 V. It was stained with Coomassie brilliant blue R-250 and distained by using a mixture of glacial acetic acid and methanol diluted with distilled autoclave water .and then bands was observe.

RESULTS AND DISCUSSION

Patients samples

10 samples of dengue patients were collected from holy family hospital Rawalpindi in which 5 are male and 5 female patients .The average age of patients is around 8-52 and maximum age was 52 and minimum age was 8years.The sample collection criteria were qualitative and qualitative positive samples along ELISA and NS1 positivity with age and gender.

Table: 2. Detail of patients obtained from holy hospital Rawalpindi.

Patients No	Age	Gender	Dengue fever type	Heamodilution	Lab number
1	52	Female	DHF	NO	102237
2	23	Male	DHF	No	101720
3	50	Male	DF	No	102276
4	26	Male	DHF	Yes	102362
5	18	Female	DHF	Yes	102359
6	28	Male	DSS	No	100497
7	21	Male	DHF	No	102146
8	32	Female	DF	No	102381
9	18	Female	DHF	No	102336
10	08	Female	DF	No	102402

Results of IgG

The results were obtained by using ELISA kit of CALBIOTECH a life science company.

Table: 3. Value of IgG and IgM

PATIENTS	IgG values	IgM values
1	1.856	0.234(-)
2	1.792	0.789(-)
3	0.622 (-)	1.778
4	1.1	0.254(-)
5	1.212	0.679(-)
6	2.168	0.129(-)
7	1.345	0.365(-)
8	0.276 (-)	1.647
9	1.365	0.682(-)
10	0.786 (-)	1.379

These values are taken from serum samples of patients that have 15 days back fever and other clinical symptoms the patients in which immunoglobulin G is present these patients are suffered from secondary infection because IgG is not detected in patients before 9 day of illness and those patients in whom immunoglobulin's M is present are suffered from primary infection.

Results for NS1:

These results were obtained by using kit of Humanism dengue NS1 Antigen test.

Table: 4. Result of NS1 Antigen

Patients No	NS1 antigen
1	+
2	+
3	_
4	+
5	+
6	+
7	+
8	_
9	+
10	_

The values of NS1 are taken from patients serum samples which have 15 days back fever When NS1 antigen is present in patients' blood or serum then two colored bands appeared in the NS1 antigen strip one band or ser appeared in the test region and one in the control region when no antigen is present in the blood or serum then no band appeared in the test region only one band appeared in control region.

Results for the SDS-PAGE:

The serum proteins were analyzed by using 12%polyacrylamide gel and different proteins of different sizes were amplified in normal patients but the protein which is present only in dengue patients not in the normal person which has a weight of around 48kda.

Table 5 Protein Sizes in Kdasynthesized by control and dengue patients

No	Control	Dengue patients
1	200kda	200kda
2	120kda	120kda
3	95kda	95kda
4	65kda	65kda
5	<65kda	<65kda
6	<65kda	<65kda
7	<65kda	<65kda
8	60kda	60kda
9	Absent	48kda
10	39kda	39kda

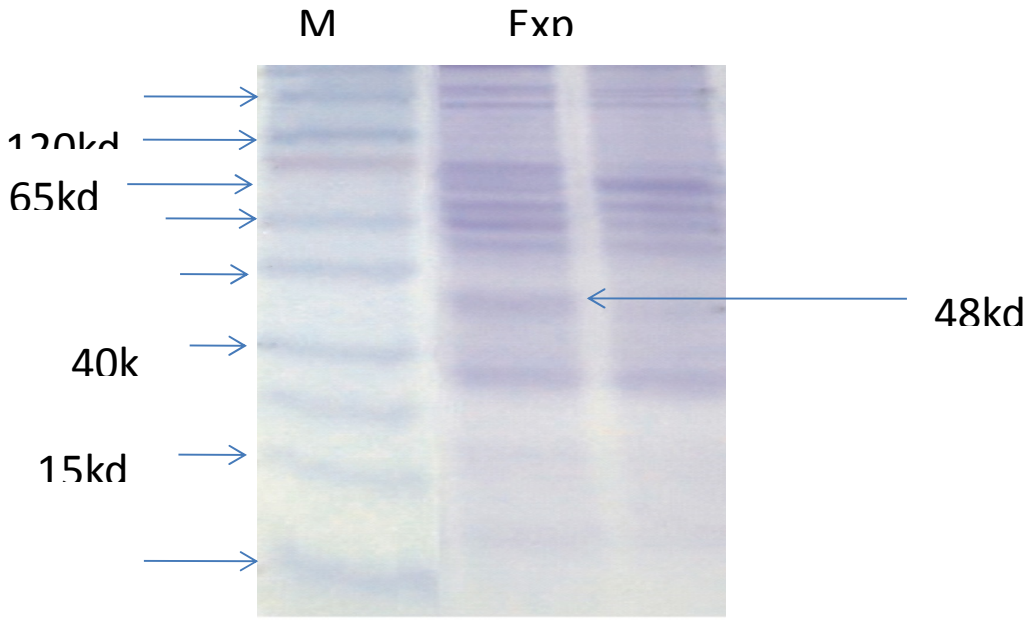

Fig. 1: SDS-PAGE analysis of serum of dengue patient and control.
Bench Mark Protein ladder (Cat No. 10747-012). Exp: infected patient Ctrl: control

DISCUSSION

In the current research focus was on the analysis of serum proteins that are responsible for pathogenesis. There are 10 predominant polypeptides observed in Aedes infected patients whose molecular weight are 200kda, 120kda,95kda,65kda, three protein having molecular weight less than 65kda,60kda,48kda and 39kda.But 9 polypeptides was observed in control with same molecular weight except 48kda. Major protein that is determined in this research was 48kda that are relatively found in dengue patients in comparison with control. But in Lee experiment Infected mosquito homogenates by SDS- Page observation 3 proteins, Mr. 49, 64 and 200 KDa from dengue virus 2 infected mosquitoes and 5 proteins, Mr. 49,50, 60, 135, and 200 KDa from dengue virus 4 infected mosquitoes were detected at higher levels than in uninfected controls. In the present research a protein having a molecular weight of 48kda was observed that is found in high amount in blood or serum of aedes infected patients by using 12% separating gel have been found similar as reported that indicated two proteins having a molecular weight of 48kda and 49kda which is found in infected patients and control samples but these proteins are over expressed in infected patients and quantitative measurement of these proteins could be considered potential diagnostic antigens to be used for detecting dengue infection in patients. In this project NS1 in patients can be confirmed by using Humasis NS1 antigen test and this viral nonstructural protein 1 could be used as an ELISA antigen to detect dengue infection in patients. The result obtained in this research found to be similar with observed a protein of 48kda in his finding which he called non structural protein1 that is found on the surface of infected cell and believed to correlate with the development of dengue hemorrhagic fever. In this study among available serological assays capture IgG and IgM ELISA and NS1antigen test are used as serological techniques for the detection of dengue virus infection and to differentiate between primary and secondary infection[9] In that serum samples were collected between day 4 and day 15 could be used for serodiagnosis and for differentiation of primary and secondary infection. We have found an easy and reliable way to define an acute primary infection If NS1 and IgG antibodies were not detected in patient's samples between day 1 and day 14 after the onset of disease. The is due to the fact that NS1 antigen and IgG would not be detected before day 9 of illness .Interestingly recently reported that the NS1antigenuse was circulating from the first day after the onset of disease[10]. Current results also in agreement with that NS1 could not be detected in acute phase serum sample taken from patients with serologically confirmed primary infection[11] .We have reported the development of an easy sensitive and specific NS1, IgG and IgM ELISA that can be reliably used for the serodiagnosis and seroepidemiological study of dengue infection.

CONCLUSION

In the current research serum protein analysis of dengue and healthy patients was carried out by using 12% Polyacrylamide gel .Prevalence of dengue hemorrhagic fever was found to be maximum (80%) and dengue fever was 20%.The recent research may be helpful for serum protein analysis of dengue patients in comparison with healthy individuals while conducting diagnostic or antiviral research and SDS-PAGE method used was found to be specific and reliable.

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