MINISTRY OF EDUCATION AND TRAINING

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CLINICAL AND SUBCLINICAL CHARACTERISTICS OF DENGUE FEVER PATIENTS AND DEVELOPMENT OF RECOMBINANT NS1 ANTIGEN POOLING 4 SEROTYPES FOR DENGUE VIRUS ANTIBODY DETECTION USING ELISA TECHNIQUE.

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PROBLEM STATEMENT

Dengue fever (DF) is an acute infectious disease caused by the dengue virus (DENV). The virus is transmitted from person to person by *Aedes spp* mosquitoes. More than one-third of the world's population lives with dengue fever (DF), an acute infectious illness caused by the dengue virus (DENV). *Aedes spp* mosquitoes spread the virus from person to person. Over 33% of the global population resides in locations susceptible to infection, with dengue fever being a significant contributor to sickness and fatalities in tropical and subtropical countries in areas at risk of infection, and dengue hemorrhagic fever plays an essential role in causing illness and death in tropical and subtropical regions.

Before 2020, the dengue fever outbreak in Vietnam followed a complex pattern, recurring every 4-5 years. In 2016, there were 109,399 cases of Dengue Fever (DF) reported across 56 provinces and cities in the country, resulting in 36 fatalities. In 2019, there were 335,056 instances reported, resulting in 55 deaths. In 2020, according to Ministry of Health data, DF was the third most prevalent infectious illness generating outbreaks, with 137,470 cases and 29 fatalities. In 2022, the country is expected to report 367,729 cases of DF and 140 deaths. By December 17, 2023, the country has registered 166,619 cases of infection, with 42 deaths.

DF is caused by four serotypes: DENV1, DENV2, DENV3, and DENV4 of the Dengue virus, which circulate differently in areas where DF is common. Differential diagnosis based on symptoms is challenging because non-specific symptoms of DF, such as fever, pain, and fatigue, often overlap with other endemic infections.

Conventional diagnostic techniques for DF involve utilizing reverse transcription polymerase chain reaction (RT-PCR) to identify dengue virus RNA or isolate the virus, followed by indirect immunofluorescence testing (IFA). Both approaches are successful within the initial five days of infection with the pathogen, but their

sensitivity diminishes as the viral load in the blood drops over time. Moreover, these conventional techniques necessitate specialized laboratory infrastructure and skilled specialists for execution, rendering them challenging to implement on a large scale within the community.

Various NS1 antigen assays have proven efficient in identifying Dengue virus in populations. NS1 antigen testing enhances diagnostic capabilities and is crucial for disease source management and vector surveillance. No NS1 antigen encompassing all four dengue virus types has been utilized, perhaps leading to missed cases of dengue virus infection. It is crucial to have an extra diagnostic approach for dengue that guarantees sensitivity, accuracy, and simplicity. We study with the following objectives based on that fact:

1. Describing the clinical and subclinical characteristics of Dengue fever patients treated at Military Hospital 103 and Military Hospital 175 in 2022.

2. Developing a recombinant NS1 antigen pool comprising four serotypes and evaluating the results of Dengue virus antibody detection using the ELISA technique.

New contributions of the thesis:

Firstly, DF's clinical and paraclinical features should be outlined based on age group and gender.

A recombinant NS1 antigen product called rAgNS1-DENV1-4 can be developed using artificial gene synthesis technology, incorporating all 4 types of Dengue virus (DENV 1-4). This product offers essential materials for producing biological products for the early detection of Dengue fever, thereby enhancing the efficacy of disease management.

Third, monoclonal antibodies specific to all 4 strains of Dengue virus can be produced for diagnostic purposes or vaccine development by utilizing recombinant antigen NS1.

Thesis structure

- A total of 127 pages, including Problem statement; 4 chapters (Chapter 1: Literature review; Chapter 2: Research subjects and methods; Chapter 3: Research results; Chapter 4: Discussion); Conclusion and Recommendations

- The thesis has 33 Tables, 27 Figures, and 174 references.

CHAPTER 1: LITERATURE REVIEW

1.1. Overview of dengue fever

Dengue fever is a sudden infectious illness caused by the Dengue virus. The virus is spread from an infected individual to an uninfected individual by mosquito bites. Aedes aegypti and Aedes albopictus mosquitoes are the primary carriers of illness. The illness is defined by fever, hemorrhage, and plasma leakage, which can result in shock and death if not rapidly and effectively treated.

Ho Chi Minh City in Vietnam reported 13,322 instances in the first eight months of 2020, the highest in the country, followed by Phu Yen with 4,898 cases. Hanoi ranks 10th with 1,993 cases. In the first eight months of 2020, serological surveillance indicated that DENV2 accounted for 51%, DENV1 for 39%, and DENV4 for 10%.

1.2. Clinical and paraclinical characteristics of dengue hemorrhagic fever

The clinical presentation of dengue virus infection varies from asymptomatic to symptoms ranging from viral infection syndrome to dengue fever, DHF, or shock syndrome. The disease can quickly progress to severe dengue, with symptoms ranging from mild, such as high fever, headache, muscle pain, and rash, to severe, such as multiple organ failure or shock. Typical clinical signs comprise elevated body temperature,

headache, eye pain, muscle and joint pain, skin rash, and hemorrhage. Patients with DF exhibit significant alterations in three primary indicators of their full blood count: white blood cell count, platelet count, and hematocrit ratio. White blood cells: Initially, there is a reduction in the white blood cell count from day 1 to day 7 of the disease. Platelets drop below 100,000 cells/mm3 throughout the clinical stage of the disease. Thrombocytopenia severity is directly related to illness severity. The hematocrit rate may be average or slightly elevated in the initial stages of the disease, accompanied by high fever, decreased appetite, and vomiting. Hemoconcentration may happen between days 3 and 7 of the illness if the Hematocrit rises more than 20% from the starting number.

Dengue hemorrhagic fever is divided into 3 levels:

- Dengue hemorrhagic fever
- Dengue hemorrhagic fever has warning signs
- Severe dengue hemorrhagic fever

1.3. Using antigens in the diagnosis of dengue fever

1.3.1. ELISA (Enzyme-Linked Immunosorbent Assay):

The ELISA method is a biological testing technique that detects and quantifies various chemicals, including proteins, peptides, hormones, and other biological compounds. There are four distinct ELISA methods: Direct ELISA, Indirect ELISA, Sandwich ELISA, and Competitive ELISA.

- The indirect ELISA approach is commonly preferred due to its adaptability and precise detection and quantification of biological components in samples. Extremely sensitive and capable of detecting microsubstances. This tool is utilized to quantify substantial quantities of various compounds in biological samples, including proteins, peptides, and hormones.
- The IgG-ELISA test detects IgG antibodies to identify previous or ongoing infections. The IgG-ELISA test lacks specificity for diagnosing Dengue serotypes due to cross-reactivity with other flaviviruses. The test

has a high sensitivity of around 82%, making IgG-ELISA useful in specific situations.

- The IgM/IgG ratio test differentiates between primary and secondary infections. The primary infection is indicated by an IgM/IgG ratio of 1.32, whereas a ratio below 1.32 signifies a secondary infection.
- Nonstructural antigen 1 (NS1) plays an essential role in the transcription of viruses into host cells. This antigen is produced in the blood of the infected patient. Therefore, NS1 is considered a vital biomarker to detect Flavivirus infection at an early stage.

1.3.2. Real-time RT-PCR:

Identifying dengue viruses using real-time RT-PCR gives more accurate results because it avoids false positives for IgM antibodies that can cross-react with other viruses of the same Flavivirus family. It is also considered the gold standard for detecting DENV infection in the early stages of infection due to its high sensitivity. In real-time RT-PCR, the viral cDNA (is synthesized from RNA from various patient samples, including plasma, blood, urine, and serum of the patient. Then, the viral RNA is transcribed reverse into cDNA) and amplifies the signal, which is read by instruments to determine whether the result is positive or negative.

1.4. NS1 antigen application in the diagnosis of dengue fever IgM/IgG antibodies and the potential of combining NS1 and IgM in rapid diagnosis

Combining NS1 and IgM tests in diagnosing dengue fever is a prerequisite to providing accurate and comprehensive information about the patient's medical condition. NS1 is a protein that appears very early in the body of people infected with the Dengue virus. Detecting NS1 can help diagnose quickly, even in the early stages of the disease, when symptoms may not yet appear or be clear.

The report from Luvira showed that the NS1 diagnostic validity report analyzed 86 sera from acute febrile patients (dengue and non-dengue fever). When compared with the results by PCR, it shows that detecting dengue NS1 by ELISA test has the highest sensitivity of 82.4% (with a specificity of 94.3%), while NS1 by quick diagnostic test (using the careUS TM Dengue Combo NS1 & IgM/IgG Kit test kit from Korea) with a sensitivity of 76.5%. IgM detection by ELISA and rapid test kit only showed a sensitivity of 27.5% and 17.9%, respectively. The combination of NS1 and IgM in the rapid test kit provides 78.4% sensitivity and 97.1% specificity.

When comparing the results of two rapid test kits using the SD Bioline Dengue NS1 Antigen biological kit and careUS Dengue IgM/IgG on patients with suspected dengue to evaluate NS1 and IgM at Nguyen Tri Phuong Hospital (Ho Chi Minh City, Vietnam). Results showed that the NS1 rapid test has sensitivity and specificity of 51.2% and 92.9, respectively, highest on day 4. The IgM antibody rapid test has a sensitivity and specificity of 21%, respectively. ,4%, 76.9%, increased gradually and was highest on day 5.

CHAPTER 2: RESEARCH SUBJECTS AND METHODS

2.1. Objective 1: Describe the clinical and subclinical characteristics of Dengue fever patients treated at Military Hospital 103 and 175 in 2022.

Research subjects

Inpatients with Dengue fever are at Military Hospital 103 and Military Hospital 175, regardless of age, gender, and socio-economic conditions.

Inclusion criteria: Based on the Guidelines for diagnosis and treatment of dengue fever, issued under Decision No. 3705/QD-BYT dated August 22, 2019 of the Ministry of Health.

Research time and location

- Research period: From January 2022 to December 2022.

- Research location: At Military Hospital 103 and Military Hospital 175

Research Methods

Research design: Cross-sectional descriptive study

Research sample size: At each hospital, apply the sample size calculation formula for descriptive research to estimate a proportion using absolute error.

$$n = Z^2_{(1-\alpha/2)} \frac{p(1-p)}{d^2}$$

With a significance level of 95%, we have Z1-α/2= 1.96; P: estimated rate of dengue hemorrhagic fever with warning signs requiring hospitalization, choose P = 31.3% according to research by Bui Vu Huy et al. in 2019, choose the absolute error d=5%, calculated n = 331 for each hospital, in reality the number of samples collected was 368 patients at Military Hospital 103 and 359 patients at Military Hospital 175.

Sampling method:

- Use convenience sampling method

- All patients hospitalized with a diagnosis of dengue fever at two hospitals who met the selection criteria were selected for the study.

- Collect clinical symptom information laboratory tests and collect patient samples.

Research content: Study to describe clinical and paraclinical characteristics in dengue fever patients at Military Hospital 103 and Military Hospital 175

2.2. Objective 2: Developing a recombinant NS1 antigen pool comprising 4 serotypes and evaluate the results of Dengue virus antibody detection using the ELISA technique.

Research subjects.

- The NS1 gene sequence of 4 types has been optimally designed using bioinformatics and artificial synthesis software at Genscript Biotech company (Piscataway, New Jersey, USA) kept in the designed

vector pJET1.2. There are two restriction enzyme cutting recognition regions: NdeI and XhoI. The design size is 471bp.

- Serum of patients diagnosed with DF at Military Hospital 103 and Military Hospital 175. Serum of healthy people participating in voluntary blood donation.

Research time and location

- Research period: From January 2022 to July 2023.

- Research location: At the Institute of Genome Research, Vietnam Academy of Science and Technology.

Research Methods

Research design: experimental research in the laboratory.

Study sample size:

Apply the sample size calculation formula to determine sensitivity and specificity using absolute error:

+ Sample size calculation formula to determine sensitivity:

$$n = \frac{z^2_{1-\frac{\alpha}{2}}\, Se(1-Se)}{d^2\, P}$$

+ Sample size calculation formula to determine specificity:

$$n = \frac{z^2_{1-\alpha/2}\, Sp(1-Sp)}{d^2\, (1-\, P)}$$

With probability threshold α = 0.05 (95% confidence level), z1-α/2 = 1.96.

Se (estimated sensitivity), in this study, choose Se = 95%;

Sp (estimated specificity), in this study, choose Sp = 90%;

d (Contrast error of Se, Sp), choose d = 5%;

P is the positive rate among test samples. In this study, it is expected that this rate will be 50% (p = 0.5).

Substituting numbers into the formulas, we have a sample size for Se estimation of 146 samples and an estimated sample size for Sp of 277 samples. 666 samples were collected and evaluated (of which 366 were

positive and 300 were negative). The gold standard for testing uses the qRT-PCR confirmatory test. Of these, 366 positive samples include 180 samples at Hospital 103 and 186 samples at Hospital 175; 300 negative samples were taken from the serum of healthy people participating in voluntary blood donation at Military Hospital 103.

Sampling method

+ Positive standard group: Patient samples of patients with dengue fever are randomly selected, with a sufficient number of 366 samples.

+ Negative standard group: Recruited by a convenient method from people who come to volunteer blood at Military Hospital 103, have no history of dengue fever, and currently have no symptoms of dengue fever. Select 300 qualified samples.

Research content

- Research on cloning and expression of recombinant antigen NS1 combining 4 virus types Dengue 1, Dengue 2, Dengue 3 and Dengue 4 on E.coli bacteria, applying Western Blot technique to identify recombinant antigen rAgNS1-DENV1-4.

- Evaluate the effectiveness of using recombinant NS1 antigen combining 4 types to detect antibodies to Dengue virus by ELISA method.

- Compare the results of using recombinant NS1 antigen of 4 types to detect antibodies to Dengue virus by ELISA method with other molecular biology techniques.

Techniques used in research

- Synthesis of recombinant NS1 antigen-specific to 4 dengue virus serotypes

The indirect ELISA method uses recombinant NS1 antigen combining 4 types to detect antibodies against Dengue virus

- Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

2.3. Errors in research

The subjects selected for the study were inaccurate due to incomplete diagnostic information.

Remedial measures: Train treatment staff and doctors to prescribe and collect enough epidemiological, clinical, and paraclinical information to diagnose dengue for patients admitted to the hospital.

Measurement error: Some test indicators (pulse, temperature, hematological, and biochemical test indicators) may be wrong and may differ due to sampling and testing techniques. Remedy: Train nursing staff and testing technicians to follow the process correctly.

Recall error: Information extracted from the patient's history and epidemiological factors may be incorrect due to inaccurate recall or interference with information from relatives. Remedy: Ask about the illness when the patient is awake and combine additional information from relatives to use the most accurate information. Check and compare information during medical examinations and inquiries.

2.4. Analyze data

- Data were entered and analyzed using SPSS 20.0 software
- Values of quantitative variables are presented as mean, standard deviation with normal distribution, median, and quartiles with non-normal distribution.
- To compare mean values, non-normally distributed variables use non-parametric tests, and normally distributed variables use t-test
- Compare rates using the Chi-Square test
- Qualitative correlation analysis, OR determination, about CI95% in the study.

2.5. Ethics in research

The study was approved by the Ethical Review Board of the National Institute of Malariology - Parasitology - Entomology (Decision No. 58/CN-VSR dated December 31, 2021) and approved by the two hospitals (Military Hospital 103 and Military Hospital 175).

CHAPTER 3: RESEARCH RESULTS

3.1. Description of clinical and paraclinical characteristics in patients with dengue hemorrhagic fever treated at Military Hospital 103 and Military Hospital 175 in 2022

Table 3.3. Signs and symptoms in research subjects

Sign and symptoms (n=727)	Số lượng	%
Fever	563	77.4
Headache	508	69.9
Body pain (muscle)	459	63.1
Athritis	352	48.4
Eye socket pain	51	7.0
Pain in the liver area	3	0.4
Nausea	273	37.6
Vomit	132	18.2
Diarrhea	87	12.0
Epistaxis	49	6.7
Bleeding tooth	178	24.5
Menorrhagia, menorrhagia	30	4.1
Vomiting blood	4	0.6
Defecation with black stools	31	4,3
Other symptoms (Urinary deficiency)	8	1.1

Among the signs and symptoms of the study subjects, fever accounted for the majority 77.4%, headache 69.9%, body pain 63.1%, joint pain 48.4%, nausea 37.6%, vomiting 18.2%. Hemorrhage symptoms include nosebleeds 6.7%, bleeding gums 24.5%, Menorrhagia 4.1%, vomiting blood 0.6%, and black stools 4.3%.

Table 3.6. Fever characteristics from disease onset according to gender

Characteristic		Gender				Total	
		Male		Female			
		n	%	n	%	n	%
Fever level (n=727)	Very high fever (5)	2	0.6	8	2,2	10	1.4
	High fever (4)	112	31.3	115	31.2	227	31.2
	Moderate fever (3)	105	29.3	128	34.7	233	32.0
	Mild fever (2)	79	22.1	56	15.2	135	18.6
	No fever (1)	60	16.8	62	16.8	122	16.8
	p	0.046					
Fever time (n=611)	From 1-3 days	129	42.9	148	47.7	277	45.3
	From 4-7 days	164	54.5	155	50	319	52.2
	Over 7 days	8	2.7	7	2,3	15	2.5
	p	0.474					
Type of fever onset (n=604)	Suddenly	213	71.5	223	72.9	436	72.2
	Wait a minute	2	0.7	3	1	5	0.8
	Unclear	83	27.9	80	26.1	163	27.0
	p	0.82					
Hot fever (n=600)	Have	259	87.5	254	83.6	513	85.5
	Are not	37	12.5	50	16.4	87	14.5

Characteristic		Gender				Total	
		Male		Female			
		n	%	n	%	n	%
	p	0.17					
Cold fever (n=603)	Have	224	75.4	229	74.8	453	75.1
	Are not	73	24.6	77	25.2	150	24.9
	p	0.868					
Fever and tremo (n=600)	Have	4	1.4	17	5,6	21	3.5
	Are not	292	98.6	287	94.4	579	96.5
	p	0.005					

The proportion of patients with high and very high fever is 32.6%; 16.8% of hospitalized patients had no fever, and the difference in fever level was statistically significant with $p < 0.05$. Most fever duration is less than 7 days, with 2.5% having a fever lasting more than 7 days. The type of fever onset is mostly sudden, 72.2%; Hot fever, 85.5%; 75.1% and chills, 3.5%. High and very high fever levels are more common in women than in men. Fever duration, onset type, fever and malaria tremors did not differ between men and women with $p > 0.05$.

Table 3.9. Bleeding characteristics by gender (n=727)

Characteristic		Gender				Total	
		Male		Female			
		n	%	n	%	n	%
Petechiae	Have	159	44.4	155	42.0	314	43.
	Are not	199	55.6	214	58.0	413	56.
	p	0.512					
Hemorrhagic plaque	Have	43	12.0	44	11.9	87	12.
	Are not	315	88. (	325	88.1	640	88.
	p	0.971					

Characteristic		Gender				Total	
		Male		Female			
		n	%	n	%	n	%
Mucosal bleeding	Have	132	36.9	128	34.7	260	35.
	Are not	226	63.1	241	65.3	467	64.
	p	0.539					
Internal bleedin	Have	5	1.4	5	1.4	ten	1.4
	Are not	353	98.6	364	98.6	717	98.
	p	0.962					

The bleeding rate between men and women is similar; 44.4% of men had bleeding, female 42%. Subcutaneous hemorrhage in male is 12%, female is 11.9%; mucosal bleeding in male is 36.9%, female is 34.7%; internal bleeding in men and women 1.4%. There was no difference in bleeding status between men and women with $p > 0.05$.

Table 3.14. Full blood count by gender

Characteristic		Gender				Total	
		Male		Female			
		n	%	n	%	n	%
Platelet count (G/ l) (n= 718)	Strong decrea (<50)	268	75.7	246	67.6	514	71.6
	Slight relief (5 149)	74	20.9	103	28.3	177	24.7
	Normal	15	3,4	15	4.1	27	3.8
	p	0.053					
	$\bar{X}$±SD (Min – Max	44.8 ± 49.0 (3-350)					
Hematocrit (l/l) (n=718)	Increase (>0.47)	177	50	39	10.7	216	30.1
	Reduced (<0.4)	16	4.5	82	22.5	98	13.6
	Normal	161	45.5	243	66.8	404	56.3
	p	<0.001					

	$\bar{X}$±SD (Min – Max	0.44 ± 0.06 (0.211-0.850)					
Hemoglobin (g/l) (n=717)	Increased (>160)	135	38.2	25	6.9	160	22.3
	Reduced (<130)	11	3.1	65	17.9	76	10.6
	Normal	207	58.6	274	75.3	481	67.1
	p	<0.001					
	$\bar{X}$±SD (Min – Max	145.7 ± 18.8 (71-210)					
Red blood ce (T/l) (n=719)	Increased (>5.4)	27	7.6	ten	2.7	37	5.1
	Reduced (<4.2)	47	13.2	121	33.2	168	23.4
	Normal	281	79.2	233	64	514	71.5
	p	<0.001					
	$\bar{X}$±SD (Min – Max	4.4 ± 0.59 (2.4-6.7)					
White blood cell (G/l) (n=718)	Increased (>10.0)	60	16.9	35	9.6	95	13.2
	Reduced (<4.0)	21	5.9	47	12.6	67	9.3
	Normal	273	77.1	283	77.7	556	77.4
	p	<0.001					
	$\bar{X}$±SD (Min – Max	7.2 ± 3.3 (1.5-22.1)					

The results in Table 3.14 show that the average urine output is 44.8 G/l, the percentage of patients with a sharp decrease in platelets is 71.6%, of which 75.7% of men have a higher reduction rate than women (67.6%); Average hematocrit 0.44 g/l, hematocrit increase rate 30.1%, of which men (50%) have a higher rate of increase than women (10.7%), statistically significant with $p < 0.05$; The average hemoglobin level is 145.7 g/l, the rate of hemoglobin abnormalities is 32.9%, of which men (41.3%) have a higher rate of abnormalities than women (24.8%). Statistically significant with $p < 0.05$; The average red blood cell count is 4.4 T/L, the abnormal rate of red blood cell count is 28.5%, and men (20.8%) have a lower abnormal rate than women (35.9%).) has statistical significance $p < 0.05$; The average white blood cell count is 7.2 G/L, and the rate of abnormal white blood cells is 22.5%, of which the rate of leukocytosis in men (16.9%) is higher than that in women (9.6%). Statistically significant with $p < 0.05$

Table 3.17. Liver function tests by gender

Characteristic		Gender				Total	
		Male		Female			
		n	%	n	%	n	%
AST (U/l) (n= 669)	Higher	9	2.8	4	1,2	13	1.9
	Moderate increas	24	7.4	31	9.0	55	8.2
	Slight increase	267	81.8	255	74.3	522	78.1
	Normal	26	8.0	53	15.5	79	11.8
	p	<0.008					
	X̄±SD (Min – Ma	209 ± 353.8 (12.8 - 5677.4)					
ALT (U/l) (n= 673)	Higher	5	1.5	2	0.6	7	1.0
	Moderate increas	16	4.9	14	4.1	30	4.5
	Slight increase	232	70.5	228	66.2	460	68.3
	Normal	76	23.1	100	29.1	176	26.2
	p	<0.222					
	X̄±SD (Min – Ma	130.8 ± 192.7 (3.1 - 2427.9)					
Bilirubin TT (µmol/l (n= 390)	Increase (>5)	51	26.2	17	8.7	68	17.4
	Normal (0-5)	144	73.8	178	91.3	322	82.6
	p	<0.001					
	X̄±SD (Min – Ma	4.3 ± 10.3 (0 - 171)					
Bilirubin T P (µmol/l (n= 508)	Increase	34	13.6	6	2,3	40	7.9
	Normal	216	86.4	252	97.7	468	92.1
	p	<0.001					
	X̄±SD (Min – Ma	12.8 ± 15.9 (1.2 - 287.8)					

The rate of moderate and high AST increase is 10.1%, for males and females it is 10.2%. The rate of moderate and high ALT elevation was 6.5%, 6.4% for male, 4.7% for female; Increased rate of total bilirubin 17.4%, average value 12.8 µmol/l, male 26.2%, female 8.7%; The rate of increased bilirubin TP was 7.9%, the average value was 4.3 µmol/l for men 13.6%, for women 2.3%. There is a statistically significant difference between AST, CPT, TT bilirubin, and TP bilirubin levels with gender at $p < 0.05$.

Table 3.2.3 . Relationship between medical history and severity of dengue fever

Anamnesis		Disease severity			OR 95% CI	p
		DF and DF with warning sign	Severe dengu	Total		
Dengue	Have	7	first	8	3.5 (0 .41 - 29.6)	0.246
	Are not	691	28	719		
	Total	698	29	727		
Hypertension	Have	33	1	34	0.71 (0.09 - 5.45)	0.750
	Are not	665	28	693		
	Total	698	29	727		
Gastritis	Have	85	5	90	1.5 (0.55 - 4.04)	0.420
	Are not	613	24	637		
	Total	698	29	727		
Hepatitis	Have	45	4	49	2.32 (0.77 - 6.96)	0.133
	Are not	653	25	678		
	Total	698	29	727		
Diabetes	Have	23	2	25	2.17 (0.48 - 9.69)	0.309
	Are not	675	27	702		
	Total	698	29	727		
Other chronic diseases	Have	103	6	109	1.5 (0.59 - 3.79)	0.384
	Are not	595	23	618		
	Total	698	29	727		

Most people with a history of chronic disease are at risk of worsening dengue fever, but this association is not statistically significant with $p > 0.05$.

3.2. Developing a recombinant NS1 antigen pool comprising 4 serotypes and evaluating the results of Dengue virus antibody detection using ELISA technique.

3.2.2. Cloning and expression of recombinant antigen NS1 combining 4 Dengue virus types 1, 2, 3, and 4 on E.coli bacteria

3.2.2.1. Transform pET22b+ – NS1 into E.coli BL21 cells

After being converted to the nucleotide sequence, the recombinant protein sequence was artificially synthesized at Genscript Biotech company (Piscataway, New Jersey, USA) with two restriction enzymes, NdeI and XhoI, at the two ends, the

artificial NS1 gene was kept in the cloning vector pJET1.2. When treated with the two upper restriction enzymes, two bands of the correct size are produced. The above band is the vector holding the pJET1.2 line with a size of 2,974 bp and the NS1 gene segment with a size of 471 bp.

Cut the NS1 gene fragment on an agarose gel to remove the gel and purify it with a GenJET PCR Purification Kit (Thermo Scientific, USA). Then, the NS1 gene fragment cut from the plasmid is connected to the pET22b+ vector (opened) using T4 ligase. The ligation mixture was transformed into E.coli BL21 competent cells. Check colonies by PCR with primer pair NS1 Fw/Rv. According to theory, the PCR product obtained is about 471 bp. PCR product results were checked on 1% agarose gel.

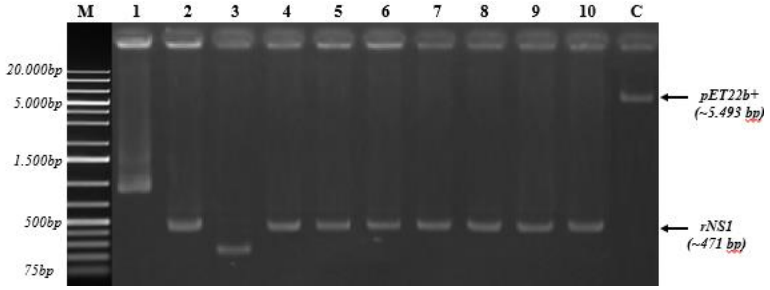
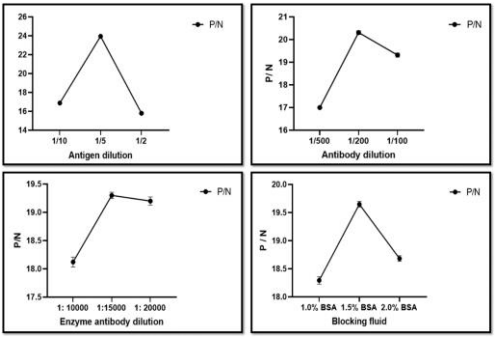


Figure 3.8. Screening of E.coli BL21 cells after transformation

Colony C (Figure 3.8) is vector pET22b+ without transformation gene (size about 5,493 bp). Colonies 2; 4; 5; 6; 7; 8; 9; 10 appears a band with a size of about 471 bp, in accordance with theoretical calculations. From the above results, it can be seen that E. coli BL21 carrying vector pET22b+-NS1 was obtained.

3.2.3.2. Optimal results of ELISA NS1 reaction



The optimal results of the indirect ELISA reaction showed: recombinant antigen rAgNS1-DENV1-4 diluted at a ratio of 1/5 (200 µg/ml), conjugate antibody diluted at 1/5000 (1µl conjugated antibodies on a total of 5 ml buffer), Figure 3.15. Optimization of indirect ELISA using recombinant NS1 protein carrying epitopes recognizing four DENV1-4 serotypes. (A) Optimization of recombinant antigen rAgNS1-DENV1-4. (B) Optimization of antibodies against dengue virus. (c) Optimization of HPR conjugated antibody. (D) Optimize the blocking concentration at 1.5% BSA for the most economical and optimal reaction (Figure 3.15).

3.2.3.3. Results of detecting IgG and IgM antibodies in patient serum samples using recombinant antigen NS1

From the above optimal results, recombinant antigens to detect IgG/IgM in the blood of patients with dengue fever using the ELISA method were applied to samples collected from two hospitals (Military Hospital 103 is 180 samples - the North and Military Hospital 175 is 186 samples - South). To determine the cutoff values of positive and negative samples, 300 healthy human serum samples without antigens and anti-NS1 antibodies of DENV1-4, were determined by real-time PCR. The results show that the average value of OD $_{450}$ $\bar{X}$ = 0.284, standard deviation SD = 0.07778. According to the formula presented

above, the cut-off value is determined to be 0.353 (Figure 3.16). Therefore, DENV 1-4 anti-NS1 antibody-positive serum samples can be identified if the OD_{450} is 0.353 or higher; otherwise, the samples may be determined to be negative.

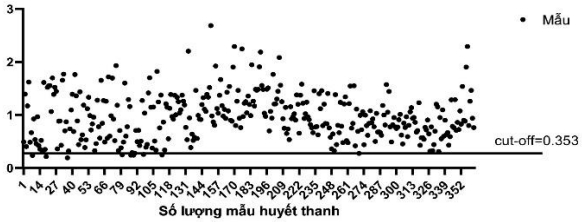


Figure 3.16. ELISA results identified dengue hemorrhagic fever-positive samples in 2 study groups. Cut-off value = 0.353.

The test to detect antibodies against NS1 of DENV 1-4 by ELISA showed results in Northern samples with 169/180 positive samples, 11/180 negative samples. The results in the South are different from those in the North; specifically, the number of positive samples is 182/186, and there are 4/186 negative samples. Thus, the evaluation results by the ELISA method showed that there were 351 true positive samples and 15 false negative cases (Table 3.31). Among 300 negative control samples, 2 false positives and 298 true negatives appeared when doing the indirect ELISA immunoassay.

Table 3.29. Sensitivity and specificity of ELISA test to detect anti-NS1 antibodies using rAgNS1-DENV1-4

ELISA	Real-time RT-PCR		Total
	(+)	(–)	
(+)	351	2	ELISA (+) = 353
(–)	15	298	ELISA (–) = 313

Total	366	300	666
Se (%)	95.90		
Sp (%)	99.33		
PPV (%)	99.43		
NPV (%)	95.21		

Applying the formula for calculating sensitivity, specificity, positive prediction, and negative prediction according to the formula in the method section shows that the indirect ELISA qualitative test set gives 95.9% sensitivity and 99.33% specificity with a cut-off threshold of 0.353. The negative predictive value was 95.21%, while the positive predictive value was 99.43%.

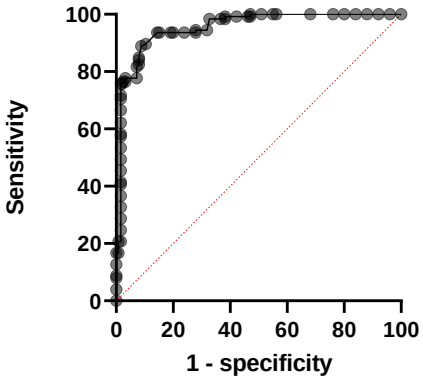


Figure 3.17. ROC curve evaluates the reliability of recombinant NS1 antigen in ELISA test

The ROC (receiver operating characteristic) chart has the vertical axis Y as the true positive rate and the horizontal axis X as the false positive rate (1 - specificity) comparing the indirect ELISA results' cut-off points. Using recombinant NS1 antigen pooling 4 types with results from the gold standard real-time RT-PCR for each patient. The points on the coordinates

represent a classification cutoff; many such points form a curve that tends to approach the asymptotic point 1. This curve always points upward, if it curves down the shows that the results are wrong and the result handling is not satisfactory. The more the line deviates to the top and left, the clearer the distinction between the two states (dengue hemorrhagic fever or not). To evaluate the reliability of a data set, observe data from the area under the AUC curve (Area Under the Curve), the larger the area, the higher the accuracy. GraphPad Prism 9.0.0 software shows the area under the AUC curve is 0.9541 with P value < 0.0001 (Figure 3.17). The AUC threshold proves that the Dengue common NS1 recombinant antigen meets the threshold. Good value in detecting IgG/IgM in patients with dengue fever.

CHAPTER 4: DISCUSSION

4.1. Describe the clinical and paraclinical characteristics of dengue fever patients treated at Military Hospital 103 and Military Hospital 175 in 2022.

This study was conducted at two Military Hospitals (Military Hospitals 103 and Military Hospitals 175), located in the North and South of Vietnam and both participate in emergency care and treatment for soldiers and people. 727 hospitalized patients were tested, diagnosed, and treated for DF. The average age of the study subjects was 36.8 years old, of which the youngest was 8 years old and the oldest was 80 years old. Most of the age distribution is concentrated from 18 to under 60 years old, accounting for 91.1%.

The symptoms of the research subjects, fever accounted for the majority of 77.4%; headache 69.9%; muscle pain 63.1%; joint pain 48.4%; nausea 37.6%, vomiting 18.2%; Symptoms of dengue warning signs include: nosebleeds 6.7%; bleeding gums 24.5%; Menorrhagia 4.1%; vomiting blood 0.6%; black stools 4.3%; Oliguria 1.1%. The functional symptoms of study participants included groups of systemic symptoms, symptoms of viral infection, symptoms of the digestive system, and symptoms related to bleeding, and a fever rate of 74, 7% is lower than other studies (96.9%) because the subjects recruited are

hospitalized patients, fever when hospitalized will gradually decrease. Patients will be treated at a dangerous stage.

Symptoms of viral infection appear at rates ranging from 7% to 63.1%, including body pain, muscle and joint pain, and eye pain. These rates are higher than those of research on the 2019 outbreak. Digestive symptoms range from 0.4% to 36.7%, with symptoms of liver pain, nausea, vomiting, and diarrhea. Loose stools, compared to the 2019 epidemic, the results of the thesis were lower, only the rate of nausea was higher, specifically in the 2019 epidemic, the rate of digestive symptoms ranged from 2.1% to 29%, 7%. Hemorrhagic manifestations range from 0.6% to 24.5%, In which the symptoms of vomiting blood and melena were at rates of 0.6 and 4.3%. This study showed lower results than in 2017 (10.1%) but higher than in 2019 (2 7%) in a survey by Nguyen Van Truong et al. evaluating two dengue epidemics in 2017 and 2019.

The rate of fever before hospitalization in men was 77.4%; Females is 77.5%; The rate of patients with high and very high fever was 32.6%; 16.8% of hospitalized patients had no fever. The difference in fever level is statistically significant, with $p < 0.05$. Most fever duration is less than 7 days, with 2.5% having a fever lasting more than 7 days. Type of fever onset: mostly sudden onset 72.2%; Accompanying fever is hot fever in 85.5%, chills in 75.1. The rate of moderate and high fever in this study is much lower than the study by Nguyen Thi Thanh Tu (2022), with the rate of moderate and high fever 99.2% assessed in 2016; 94.4% evaluated in 2017, the explanation for this may be due to the way of selecting research subjects in the thesis, which are hospitalized patients with warning signs. For dengue fever, the common characteristic is that fever will decrease after 3 days because This reduces the rate of fever. The number of patients with fever lasting more than 4 days in this study is 54.7%, much lower than the study Nguyen Thi Thanh Tu (2022) 93.8%.

The rate of petechiae between men and women is similar: male hemorrhage 44.4%; female 42%. Hemorrhagic plaques in men 12%, women 11.9%; mucosal bleeding in men 36.9%, women 34.7%; internal bleeding in men and women: 1.4%. When compared with Nguyen Thi Thanh Tu's research in two periods in 2016 and 2017, the results of this study are lower, specifically 83% in 2016 and 84.4% in 2017; but mucosal bleeding is higher (35.8%) when calculating the sum of 2016 and 2017 is 32.6%; The rate of internal bleeding in the study (1.4%) was much lower than 17.4% in this study. The lower rate may be due to the outbreak of dengue fever in 2016-2017, which led to the rate of admission to hospitals for treatment, which often prioritized patients with more severe symptoms, so the symptoms of patients were often more severe than others. With years of sporadic epidemics.

The average platelet count is 44.8 G/L, and the proportion of patients with platelets decreased sharply 71.6%, of which men 75.7%) had a higher reduction rate than women (67.6%); Average hematocrit 0.44 g/l, hematocrit increase rate 30.1%, of which men (50%) have a higher rate of increase than women (10.7%), statistically significant with $p < 0.05$; The average hemoglobin level is 145.7 g/l, the rate of hemoglobin abnormalities is 32.9%, of which men (41.3%) have a higher rate of abnormalities than women (24.8%). Statistically significant with $p < 0.05$; The average red blood cell count is 4.4 T/L, the abnormal rate of red blood cell count is 28.5%, and men (20.8%) have a lower abnormal rate than women (35.9%).) has statistical significance $p < 0.05$; The average white blood cell count is 7.2 G/L, and the rate of abnormal white blood cells is 22.5%, of which the rate of leukocytosis in men (16.9%) is higher than that in women (9.6%). Statistically significant with $p < 0.05$. This study has some indicators similar to the study by Arshad et al. in 2022, such as hemoglobin 137.2 g/L, hematocrit 0.41 L/L, and average white blood cell count 6.86 G/L. However, the platelet count was higher, at 145.23 G/L. Compared with the study of Nguyen Van Truong et al in 2020, it shows that the values of red blood cells and hematocrit are similar

to this study. However, the number of platelets in this study is lower, but the number of white blood cells is higher. The moderate and severe AST elevation rate is 10.1% in men and about 10.2% in women. The rate of moderate and severe ALT elevation was 6.5%, 6.4% for men and 4.7% for women. The Increased rate of total bilirubin was 17.4%, with an average value of 12.8 µmol/l, male 26.2%, female 8.7%. The rate of increased total bilirubin was 7.9%. The average value was 4.3 µmol/l for men, 13.6%, for women, 2.3%. There is a statistically significant difference between AST, CPT, direct bilirubin, and total bilirubin levels with gender at $p < 0.05$. The enzyme indexes AST ALT, and total bilirubin in the thesis study are much higher than the study of Akash Khetpal et al. (2021) in which AST, ALT, and total bilirubin are 54 U/L, 28.5 U/L, and 9.5 g/L. The rate of liver damage is higher than the study by Nguyen Van Truong et al in 2017 and 2019, with rates of 77.9% and 75.53%. This study showed AST and ALT results like Ren et al (2022) 94.0 U/L and 110 U/L.

Most people with a history of chronic disease are at risk of worsening dengue fever disease, but this association is not statistically significant with $p > 0.05$. Patients with a history of dengue fever, hepatitis, and diabetes all have a risk of worsening dengue with $OR > 2$. However, this combination was not statistically significant in this study. In clinical practice, patients with such a history need to be paid attention to during treatment care for patients in this group. This study also showed results similar to those of Tran Thi Van Anh's analysis. There was no significant association between people with underlying diseases and those without, leading to increased severity of dengue.

4.2. Developing a recombinant NS1 antigen pool comprising 4 serotypes and evaluating the results of Dengue virus antibody detection using ELISA technique.

In a 2019 study by Das et al., the research team described a simple and effective method to express NS1 in E.coli, which can potentially be

used to develop monoclonal antibodies. And dual specificity for point-of-care diagnostics. The optimized, full-length synthetic NS1 gene of E. coli serotype 1 (DENV-1) was successfully cloned and expressed at very high levels as inclusions. The NS1 protein was successfully purified and refolded by affinity as recombinant NS1 (rNS1) protein in E. coli, and the bacterial culture yield was 230–250 mg/L. The rNS1 protein was used to immunize hybridoma-grown mice. Thus, it can be seen that E. coli has great potential to clone and express the NS1 gene specific to all 4 dengue virus serotypes. Therefore, in this study, E. coli was selected to express and clone the NS1 gene specific to all 4 designed Dengue virus serotypes.

The process of transforming plasmid DNA into E. coli cells was performed using the heat shock method, in which, before heat shock, the cells were loosened in CaCl2 solution to increase the ability to accept plasmid DNA. Using the E. coli BL21 bacterial strain, this study successfully transformed the recombinant expression vector pET-22b(+)/NS1-DENV1-4 into cells. Transformed cells were positively selected on agar medium containing ampicillin antibiotic. Through PCR testing with primer pairs T7 F and T7 R and Sanger sequencing of the obtained gene segment and checking that sequence with the designed gene sequence, the results are Figures 3.7 and 3.8 with the size of the nuclear gene segment product. Up to about 471bp, the sequence completely matches the sequence of the designed NS1 gene segment. These results demonstrate that the NS1-DENV1-4 gene expression construct in E. coli BL21 cells was successfully established.

To date, real-time RT-PCR has been recognized as the gold standard in DENV diagnosis and serotyping, however, there are still some disadvantages, including false negative and false positive results and especially It is necessary to be equipped with expensive machinery as well as a very specialized laboratory and a team of technicians with extensive training in molecular biology techniques. This makes implementation difficult at the grassroots and community levels,

especially in complicated epidemic areas. Therefore, there is a great need for a simple, easy-to-deploy tool to make diagnoses faster, thereby better-controlling dengue fever.

Preliminary assessment shows that using recombinant antigen rAg-NS1-DENV1-4 shows high similarity with 2 commercial rapid test kits for IgM/IgG testing. When the disease is over 20 days, the sensitivity still reaches 89%, but at this stage, it will be easily confused with IgG antibodies of other diseases in the flavivirus genus (Zika, Chikungunya, Japanese Encephalitis,). Therefore, many testing methods should be combined with clinical and paraclinical symptoms in cases with a long onset time to conclude the disease. In the literature, the rate of false positive NS1 tests is only allowed to range from 0.5% to 2.0%. The results of this study show that the ELISA technique using the recombinant antigen rAgNS1-DENV1-4 has sensitivity, specificity, positive prediction and negative prediction from 95.21 - 99.43, lower than compared to the value determined using the RT-PCR method.

CONCLUSION

1. Clinical and subclinical characteristics of Dengue fever patients treated as inpatients at Military Hospital 103 and Military Hospital 175 in 2022

Research on 727 people with dengue hemorrhagic fever hospitalized for treatment at Military Hospital 103 and Military Hospital 175 during the period from January 2022 to December 2022 showed:

- Clinical features:

+ Severe Dengue fever accounted for 3.98%, with a Dengue shock syndrome rate of 1.3%.

+ Fever characteristics: Upon admission, 77.4% presented with fever, of which 32.6% had high to very high fever; 85.5% experienced

hot fever; 75.1% had a fever with chills; and 3.5% had shivering fever. ever duration: 45.3% for 1-3 days, 52.2% for 4-7 days, and 2.5% for over 7 days.

+ Hemorrhagic manifestations: Petechiae accounted for 43.2%, purpura for 12.0%, mucosal bleeding for 35.8%, and visceral bleeding for 1.4%.

+ Symptoms: Headache (69.9%), body aches (63.1%), joint pain (48.4%), eye socket pain (7.0%), nausea (37.6%), vomiting (18.2%), vomiting blood (0.6%), black stool (4.3%), diarrhea (12%).

+ Individuals over 60 years old were at higher risk of severe Dengue fever. Some risk factors such as previous Dengue fever, hepatitis, and diabetes, increased the severity of Dengue fever, but the combined effect was not statistically significant.

- Subclinical features:

+ Decreased platelet count accounted for 96.3%; increased Hematocrit for 30.1%; increased (AST) and alanine aminotransferase (ALT) levels for 88.2% and 73.8%, respectively; increased total and direct bilirubin for 17.4% and 7.9% respectively.

+ Abnormalities in albumin (27.3%), glucose (52.8%), urea and creatinine (16.8%), and C-reactive protein (CRP) (25.6%) were observed.

+ Serotype identification via quantitative reverse transcription-polymerase chain reaction (qRT-PCR) detected all 4 serotypes evenly distributed at both hospitals, with DENV 1 and 2 being the most common and DENV 3 and 4 less prevalent in the study samples.

2. Development of recombinant NS1 antigen pool comprising 4 serotypes and evaluation of Dengue virus antibody detection efficacy using ELISA technique

- Recombinant NS1 antigen pool for 4 Dengue virus serotypes was successfully synthesized in E. coli cells with purity ≥ 95%, concentration of 0.347mg/ml, and antigenicity confirmed via Western blot.

- Utilizing the recombinant NS1 antigen pool for Dengue virus antibody detection via indirect ELISA in a laboratory setting achieved a sensitivity of 95.9%, specificity of 99.33%, positive predictive value of 95.21%, and negative predictive value of 99.43%.

RECOMMENDATIONS

1. Expressing recombinant NS1 protein in different expression systems is necessary to create a protein with the highest purity and activity.

2. It is necessary to continue researching the stability of recombinant antigens combining 4 types of Dengue virus

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