Molecular Detection of Dengue Virus Outbreak in Jhapa District, Nepal

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A dissertation submitted
in partial fulfillment of the requirements for the award of the degree
of Master of Science in Zoology with special paper Parasitology

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April 2024

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Declaration

I hereby declare that the work presented in this dissertation "Molecular detection of dengue virus outbreak in Jhapa district, Nepal" has been done by myself, and has not been submitted elsewhere for the award of any degree. All sources of information have been specifically acknowledged by reference to the author(s) or institution(s).

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This is to recommend that the dissertation entitled "Molecular detection of dengue virus outbreak in Jhapa district, Nepal" has been carried out by Niten Bharati for the partial fulfilment of Master's Degree of Science in Zoology with special paper Parasitology. This is his original work and has been carried out under my supervision. To the best of my knowledge, this dissertation work has not been submitted for any other degree in any institutions.

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Certificate of acceptance

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Abstract

Dengue is prevalent throughout Nepal, with Jhapa district in Koshi province consistently affected by the disease. Despite the district's enduring dengue endemicity, there remains a lack of data regarding the virological characteristics of the illness. To address this gap, this study initiated a molecular surveillance study of circulating dengue viruses (DENV) to ascertain the prevailing virus serotypes. In Jhapa district, a cross-sectional study on dengue molecular surveillance was undertaken. Sera were obtained from patients suspected of having dengue who visited hospitals. Diagnosis was carried out utilizing a rapid diagnostic test (RDT) kit for detecting dengue NS1 antigen and IgG/IgM antibodies. Dengue serotyping was conducted using dengue RT-PCR. Additionally, hematological and demographic data of patients were collected and subjected to analysis. A total of 290 serum samples were obtained from individuals suspected of having dengue fever clinically in 2022. Among these, 46 samples, accounting for 15.9%, tested positive for NS1, IgM, and IgG antibodies via RDT. The study in 2023 involved 66 samples, showing a higher proportion of seropositivity among males (37, or 56%) compared to females. A molecular analysis was conducted on 60 samples initially identified as serologically positive, revealing that 46 of these samples tested positive through RT-PCR. This analysis showed that DENV-2 was the most common type (67.4%), followed by DENV-3 (28.3%) and DENV-1 (4.3%). None of the samples contained DENV-4. To the best of our knowledge, this report is the first to detail the circulating serotypes of dengue viruses in Jhapa District. This contribution has the potential to enhance the exploration of dengue epidemics and deepen our understanding of their pathogenesis.

शोध सार

डेङ्गु नेपालभरि नै व्याप्त छ, कोशी प्रदेशको झापा जिल्ला डेङ्गु रोगबाट निरन्तर प्रभावित हुँदै आएको छ । यस जिल्लामा डेङ्गुको प्रकोप कायमै रहे तापनि यस रोगको भाइरोलोजिकल विशेषताहरूको बारेमा तथ्याङ्कहरुको अभाव छ । यस अभावलाई सम्बोधन गर्न, यस अध्ययनले प्रचलित भाइरस सेरोटाइपहरू पत्तालगाउन सर्कुलेटिङ डेङ्गु भाइरस (डीईएन्भी) को आणविक अध्ययन सुरु गरिएको थियो । झापा जिल्लामा डेङ्गुको आणविक निगरानीसम्बन्धी क्रस सेक्शनल अध्ययन गरिएको थियो । अस्पतालहरू भ्रमण गरेका डेङ्गुको आशंका गरिएका बिरामीहरूबाट सेरा प्राप्त गरिएको थियो । डेङ्गु एनएस वान् एन्टिजेन र आइजिजी/आइजीएम एन्टिबडी पत्ता लगाउन र्यापिड डाइग्नोस्टिक टेस्ट (आरडिटी) किटको प्रयोग गरी रोग पत्ता लगाइएको थियो । डेङ्गु आरटी-पीसीआरको प्रयोग गरेर डेङ्गु सेरोटाइपिङ गरिएको थियो । थप रूपमा, बिरामीहरूको हेमेटोलोजिकल र जनसांख्यिकीय डेटा सङ्कलन गरिएको थियो र विश्लेषण गरिएको थियो । सन् २०२२ मा डेङ्गु ज्वरोको आशङ्का गरिएका व्यक्तिहरूबाट २९० वटा सीरम नमूनाहरू लिइएको थियो । तीमध्ये ४६ (१५%) नमूनामा आरडिटी विधिबाट एनएस वान्, आइजीएम र आइजिजी एन्टिबडी पोजिटिभ देखिएको थियो । सन् २०२३ मा गरिएको अध्ययनमा ६६ वटा नमूना समावेश गरिएको थियो, जसमा महिलाको तुलनामा पुरुष (३७ वा ५६ प्रतिशत) मा सेरोपोजिटिभिटीको अनुपात बढी देखिएको थियो । सुरुमा ६० वटा नमूनामा आणविक विश्लेषण गर्दा ४६ वटा नमुनामा आरटी-पीसीआर विधिबाट पोजिटिभ देखिएको थियो । यो विश्लेषणले डीईएनभी -२ सबैभन्दा सामान्य प्रकार (६७.४ प्रतिशत), त्यसपछि डीईएनभी -३ (२८.३ प्रतिशत) र डीईएनभी -१ (४.३ प्रतिशत) देखियो । कुनै पनि नमूनामा डीईएनभी-४ थिएन । हामीलाई थाहा भएसम्म यो रिपोर्ट झापा जिल्लामा डेङ्गु भाइरसको सर्कुलेटेड सेरोटाइपको विस्तृत विवरण दिने पहिलो रिपोर्ट हो । यो अनुसन्धाने डेङ्गु महामारीको अन्वेषण बढाउन र रोगको बारेमा हाम्रो बुझाइलाई गहिरो बनाउने क्षमता राख्छ ।

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List of abbreviations

Abbreviated form	Details of abbreviations
WHO	World Health Organization
DENV	Dengue Virus
DF	Dengue Fever
DHF	Dengue Hemorrhagic Fever
DSS	Dengue Shock Syndrome
DVI	Dengue Virus Infection
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
ELISA	Enzyme-linked Immunosorbent Assays
CBC	Complete Blood Count
WBC	White Blood Cells
IgM	Immunoglobulin M
µl	Micro liter
ml	Milliliter
RNA	Ribonucleic Acid
i.e.	Id Est
STIDH	Shukraraj Tropical & Infectious Disease Hospital
NS1	Non-structural Protein 1
cDNA	Complementary Deoxyribonucleic Acid
EDCD	Epidemiology and Disease Control Division

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1. Introduction

1.1 Background

Dengue fever (DF) is regarded as the most significant viral disease transmitted by arthropods to humans, posing a risk of infection to more than half of the global population across more than 100 countries (WHO, 2009). Dengue event incidence, fatalities, and Disability-adjusted life years (DALYs) grew significantly worldwide between 1990 and 2019 (Yang et al., 2021). More than five million cases of dengue have been reported since the beginning of 2023 due to ongoing transmission and an unexpected spike in the number of cases. Over 5000 deaths have been reported in over 80 countries/territories and five WHO regions: Africa, Americas, South East Asia, Western Pacific, and Eastern Mediterranean (WHO, 2023). Over 125 countries are known to be dengue endemic and dengue transmission is present in every World Health Organization (WHO) region (Murray et al., 2013). Dengue infections occur in Southeast Asian countries on an average every year, resulting in 5906 deaths and an economic burden of US$950 million (Shepard et al., 2013). Due to migration and international travel, dengue, a disease that primarily affects tropical and subtropical regions, is now spreading over the entire planet. More people die from dengue than from any other arboviral illness (Leung-Chen, 2008). There are around 2 billion people who could contract dengue fever (Dung et al., 1999). Despite the fact that classical dengue is not particularly dangerous, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), which often develop after the initial dengue infection, can seriously affect a person's health and even result in death. Abdominal discomfort, headaches, rash-like flashes, vomiting, and nausea are among the main dengue symptoms. The dengue virus is spread by *Aedes* mosquitoes. People's involvement in dengue prevention is crucial since *Aedes* mosquitoes are primarily attracted to humans, reside close by, and spawn in water containers. Understanding attitudes and behaviors surrounding both dengue sickness and mosquito control is crucial for promoting effective community involvement in dengue prevention efforts (Crabtree et al., 2001).

The World Health Organization (WHO) has recently developed new clinical recommendations to categorize dengue severity, although serological, virological, and molecular biology testing are still necessary to conclusively identify dengue virus (DENV) infection. Many endemic countries struggle with laboratory diagnosis of dengue due to a lack of materials, high costs, or long wait times for results. In order to create a clinical

prediction algorithm that can distinguish dengue cases from those of other febrile illnesses, it is crucial to identify distinctive clinical and laboratory markers that appear during the early fever phase of the illness. Furthermore, identifying patients who are in danger of developing severe dengue is essential for timely supportive care, which can lower the risk of mortality to less than 1% (Guzmán & Kourí, 1996; Salje et al., 2012).

DENV-1, DENV-2, DENV-3, and DENV-4 are four dengue viral serotypes that are closely related and cause dengue symptoms. Three different types of symptoms can be used to categorize the dengue infection: DF, DHF, and DSS. In contrast to cross-immunity, which is only transient and partial, a host will have lifetime immunity against dengue after recovering from the illness. The host will be at a very high risk of getting a severe dengue infection from a different serotype after contracting one of the serotypes (Gubler, 1998; WHO, 2009; Barmak et al., 2011).

As DF/DHF epidemics have occurred in India, DF/DHF has been thought to pose a potential public health danger to Nepal (Gupta et al., 2006). Nepal is more vulnerable to the severity of dengue virus infection (DVI) due to its proximity to India on its eastern, western, and southern borders. The open border makes it simple for infected people to pass, and the vector could spread the infection. In 2004, there was a report of the first DF case in Nepal (Pandey et al., 2004). Further, the first DEN-2 strain of Nepali origin was discovered in a Japanese tourist who had visited Nepal and later acquired DF upon returning to Japan. In October 2006, following the DF/DHF epidemic in India, an outbreak of the disease occurred in Nepal (Takasaki et al., 2008).

The diagnosis of dengue can be made using clinical laboratory tests in addition to the description of clinical symptoms. The primary indicators of dengue often include fever, nausea, vomiting, a rash, as well as discomfort such as aches and pains (which may manifest as eye pain, usually felt behind the eyes, muscle soreness, joint discomfort, or bone pain) (CDC, 2023). A complete blood count (CBC), particularly the white blood cell (WBC), platelet, and hematocrit counts, is one of several clinical assays. Albumin, liver function, and urine testing are additional helpful diagnostic techniques (WHO, 2009). Basic serologic and dengue-specific testing is frequently used to confirm probable dengue infections. As soon as a suspected illness manifests, a blood sample from the acute phase should always be obtained. Non-hospitalized case follow-up is challenging and time-consuming. Consequently, it is advised that before hospital discharge, individuals who are hospitalized

should have a second blood sample taken. Direct virus isolation, which may be carried out using either mosquito cell cultures or mosquito inoculation, is useful for determining the serotype of the infecting virus by RT-PCR. The fundamental serologic test is the IgM ELISA, which looks for anti-dengue-neutralizing IgM antibodies (Gubler, 1998; WHO, 2009). Due to a lack of diagnostic resources, dengue diagnosis and treatment in Nepal are based on the patient's clinical symptoms (Pandey et al., 2004). Dengue virus has been present in Nepal for an extended period.

The prevalence of DF in Nepal has been recorded in a number of research. The seroprevalence changes over time; it was 10.4% in 2006, 29.3% in 2008, 35% in 2010 and 19.3% in Chitwan and Dang in 2013 (Malla et al., 2008; B. D. Pandey et al., 2013; Shah et al., 2012; Shrestha et al., 2016). In 2022, laboratory-confirmed dengue cases accounted for 74.5% of all suspected cases (Rimal et al., 2023). Although Nepal has experienced endemic dengue since 2006, the number of cases has steadily risen. In 2023, a collective total of 51,243 dengue cases were documented across all seven provinces (EDCD, 2023). This suggests that the country is experiencing rapid geographical expansion of dengue. To stop Nepal from being more at risk from DVI, disease management must be done properly.

1.2 Statement of problem

In Nepal, dengue has been classified as a national concern for decades. Every year, a portion of the national budget is allocated for campaigns. There are frequent dengue outbreaks in the country, but molecular studies have been limited, leading to an insufficient understanding of serotypes. Nepal has experienced several dengue outbreaks in recent years, with an increasing number of cases and deaths reported each year. Research in dengue in Nepal is essential for improving our understanding of the virus and its transmission, informing prevention and control strategies, and contributing to the global effort to combat dengue. This study aims to investigate the prevalence and molecular epidemiology of dengue as well as its changing trends.

1.3 Objectives

1.3.1 General objective

- To investigate the molecular detection of dengue virus outbreak in the Jhapa district.

1.3.2 Specific objectives

- To find out the distribution of dengue virus serotypes circulating in the jhapa district.
- To identify the demographic and hematological features of serologically confirmed dengue cases.

1.4 Research questions

- Which serotype of dengue virus is most prevalent in Jhapa district?
- Is there difference in the distribution of demographic and hematological parameters among dengue patients?

1.5 Significance of the study

This study is pioneering in revealing the circulating serotypes of dengue virus within the Jhapa district of Nepal. The study marks the first investigation of dengue virus serotype circulation in 2023 in Nepal, with no prior research published on the topic. This research can help to identify the high-risk for dengue transmission, which can guide the development of surveillance and prevention programs. It has significant implications for national programs and local health services, by providing important information for the development of effective prevention and treatment strategies, as well as informing public health education and resource allocation decisions. The results from the research can be published in scientific journals, which can be assessed by researchers, clinicians, and public health professionals. The results will also be disseminated through newspapers as well as through social media platforms. This can help raise awareness of dengue and its impact and educate the public on prevention and control measures.

1.6 Limitations of the study

- Cross reactivity of serological tests with other flavivirus.
- Sample does not reflect the general population.
- Insufficient sample size for statistical measurement.
- Time constraints.

2. Literature review

2.1 Serotype circulation and distribution of dengue virus:

Several cases of dengue virus serotype 3 were characterized in Brazil, 15 years after the last outbreak of this serotype in the country (Naveca et al., 2023). During the investigation carried out in Brazil in 2021 and 2022, researchers retrieved nearly complete nucleotide sequences of 57 DENV-1 viral genomes alongside four DENV-2 genomic sequences (Souza et al., 2023). In Malaysia, DENV-3 and DENV-2 were the primary serotypes in circulation during 2017–2018 and 2019–2020, respectively. However, a transition in serotype dominance occurred from 2021 onwards, with DENV-4 emerging as the predominant circulating serotype (Suppiah et al., 2023). In a 2022 study involving both children and adults in Indonesia, ten cases tested positive for dengue virus, with DENV-1 being the prevailing serotype at 70%, followed by DENV-2 at 20%, and DENV-4 at 10% (Datu et al., 2023). Throughout the study period in Guangzhou, all four serotypes of dengue virus were observed, but their simultaneous circulation was only observed in 2010, 2016, and 2018 as per. DENV-1 was the most commonly identified serotype over the decade, except for 2010 and 2012 when DENV-4 and DENV-3, respectively, were predominant (Jiang et al., 2023). Between 2013 and 2016, DENV-1 and DENV-2 dominated in Bangladesh, while DENV-3 remained absent (Muraduzzaman et al., 2018). From 2017, DENV-3 resurfaced and became the primary circulating serotype, replacing DENV-2 (Rahim et al., 2021). By 2019, DENV-3 accounted for the majority of cases, surpassing DENV-1 and DENV-2 (Titir et al., 2021). In Pakistan, dengue serotyping revealed a prevalence of DENV-2 at 62%, with DENV-1 following closely at 37%. Additionally, one instance of DENV-3 was identified in the recent study(Rahim et al., 2021). Four separate outbreaks occurred in Sri Lanka during the monsoon seasons, primarily driven by the DENV-2 cosmopolitan genotype, with the exception of a significant outbreak in 2019, which was predominantly caused by DENV-3 genotype I(Maduranga et al., 2023). In Pakistan, out of 373 samples tested, 73% were positive for dengue virus infection, with males comprising 61.9%, and the 2021 outbreak demonstrated complete predominance of the DENV-2 serotype (99%), with only one case of DENV-1 detected (Hakim et al., 2023). Of the RNA-positive patients (n = 95) included in the study, DENV-2 (70.54%) emerged as the predominant circulating serotype, followed by DENV-3 (18.94%), DENV-1 (6.31%), and DENV-4 (4.21%) (Verma et al., 2022).

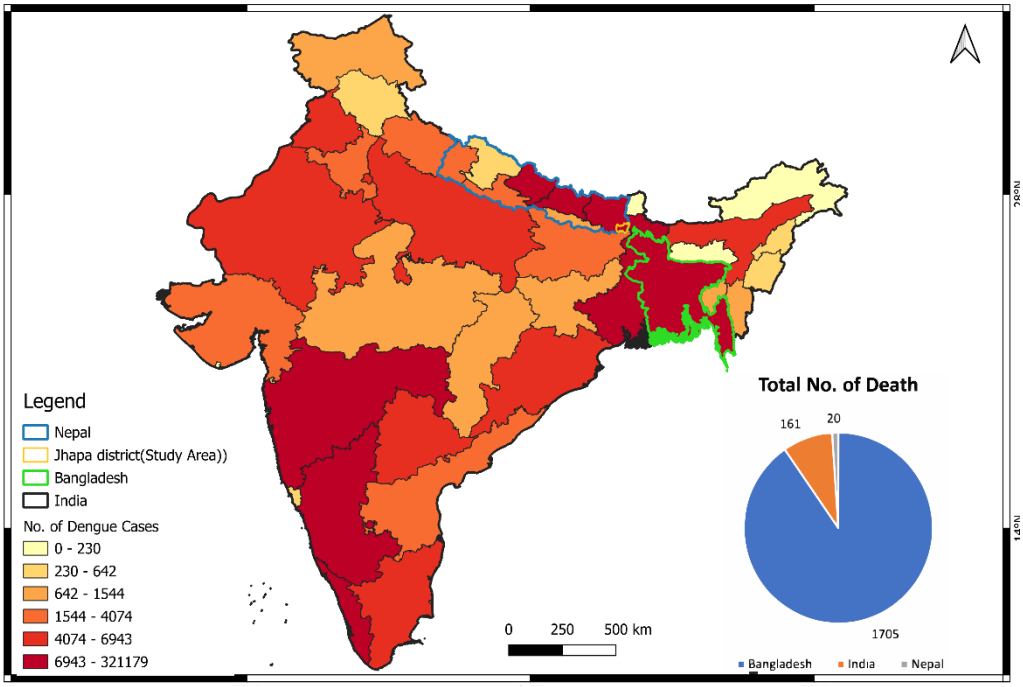

Figure 1. Map of Southeast Asia (India, Nepal, and Bangladesh) showing the number and death cases due to dengue virus (Source: DGHS, 2024; EDCD, 2023; NCVBDC, 2024)

During the study in Kathmandu in 2023, the circulation of three serotypes of Dengue virus (DENV-1, DENV-2, and DENV-3) was confirmed, with DENV-4 not detected. Out of the patients who tested positive for DENV PCR (n = 29), the most prevalent serotypes were DENV-1 (57.1%) and DENV-3 (32.1%) (Rimal et al., 2023). RT-PCR testing conducted at STIDH in 2019 revealed that out of 195 samples analyzed, 57.0% tested positive for dengue viral RNA. Among the positive cases, DENV-2 (63.1%) emerged as the predominant serotype, with two samples testing positive for DENV-3 (Poudyal et al., 2021). According to the findings of another study, 34.8% of the samples tested positive for the DENV-2 serotype (Tun et al., 2021). From the 15-dengue virus (PCR) positive samples acquired in 2017, three displayed positivity for DENV-1, twelve for DENV-2, one for DENV-3, yet no indication of DENV-4 was discerned within any of the samples (Prajapati et al., 2020). During 2010, the primary serotypes detected were DENV-1 (64.4%) and DENV-2 (27.8%), with two cases of DENV-3 and five cases of DENV-4 also confirmed (Dumre et al., 2017). The serotyping of 75 serum samples from patients with fever lasting less than 4 days in 2016 revealed that the dengue outbreak in Nepal that year was mainly, if not entirely, due to DENV-1. This marked a change from the dominant DENV-2 serotype observed during the 2013 epidemic (Khetan et al., 2018). In 2013, serotyping using semi-nested PCR

revealed an amplification of 119 base pairs, confirming the virus to be serotype 2 (Gupta et al., 2015).

The 2010 pandemic may have been caused by the DENV-1 serotype, according to research done at the Sukraraj Tropical and Infectious Disease Hospital (STIDH) in Kathmandu, the capital of Nepal (B. D. Pandey et al., 2013). In the low-land Terai region, specifically in nine districts, all four DENV serotypes were observed to be circulating (Malla et al., 2008). While the initial reported case from the Japanese traveler in 2004 was identified as serotype-2, it's important to note that serotyping of the virus is not conducted on an annual basis in Nepal (Takasaki et al., 2008).

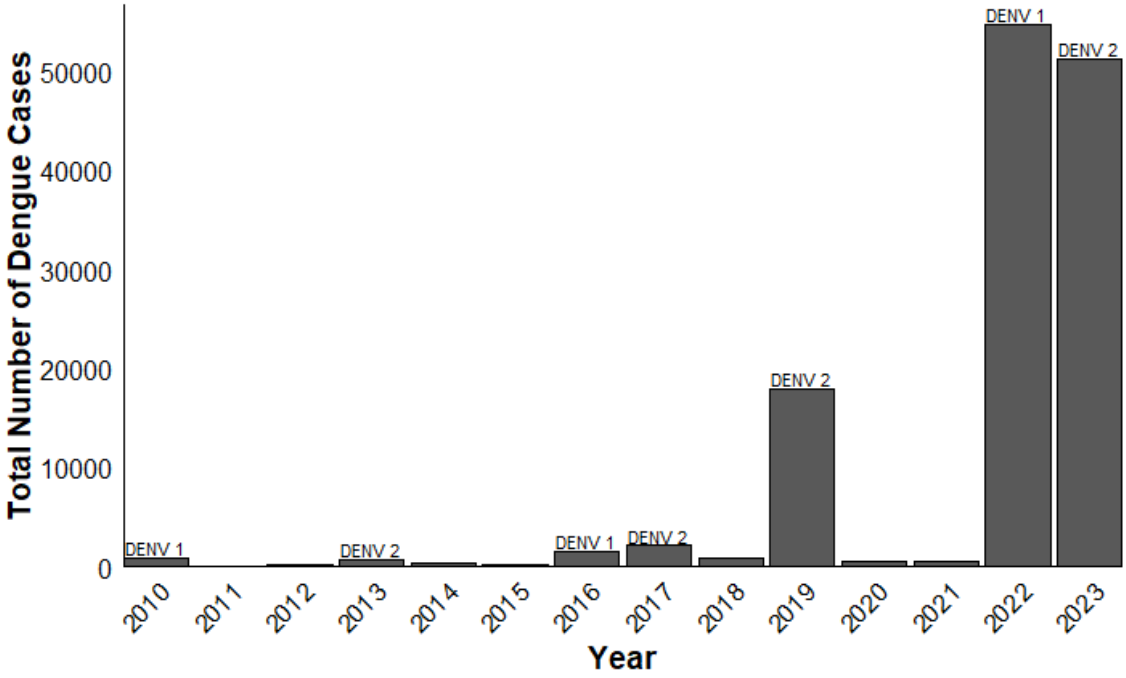

Figure 2. Number of dengue cases reported in Nepal during 2010–2023 with dominant serotype (source: EDCD, Nepal)

2.2 Demographic and hematological parameters in dengue virus infected patients:

Dengue is a severe feverish illness that appears within three to ten days following a mosquito bite carrying the dengue virus (Teixeira & Barreto, 2009). A study in Brazil found that there weren't significant differences between males and females among the people affected, with males comprising 50.8% and females 49.2%. The younger age groups, ranging from 4 to 43 years old, were the most impacted (Souza et al., 2023). During the entire study duration spanning from 2017 to 2022 in Malaysia, 75 specimens testing

positive for the DENV-4 serotype were detected, with patients' ages ranging from 2 to 80 years and averaging 36.1 years. Most patients were adults over 18 years old (82.7%), and the majority were male (56.0%) (Suppiah et al., 2023). In China, the risk of infection was not influenced by gender. Despite the majority of cases (57.88%; 25,692 out of 44,385) occurring in individuals aged 20–49 years, the infection risk exhibited a rising trend with advancing age after adjusting for gender and year (Jiang et al., 2023). Studies in Bangladesh consistently demonstrate a strong male predominance in dengue outbreaks, with men comprising approximately twice as many cases as women. The male-to-female ratio reaches up to 2.7 in some instances. Adolescents and adult populations showcase this trend, although there is no significant difference in pediatric groups (Khan et al., 2021). The majority of cases in the 2016, 2018, and 2019 outbreaks in Bangladesh were among older adolescents and young adults, with individuals aged 21-40 accounting for 55%, 65%, and 50% of the cases, respectively (Hasan et al., 2021; Pervin et al., 2017; Sultana et al., 2019). In a study conducted in Pakistan, among the PCR-positive cases, males were more commonly infected (63%) compared to females (37%) both in 20, with the mean age of dengue-positive patients being 30.75 (±12.4) years, averaging 29 years for males and 33 years for females (Rahim et al., 2021). In 2022, a study enrolled 170 pediatric patients admitted to hospitals in Kolkata, India, suspected of having dengue and aged 12 years or younger, with fever persisting for up to 5 days. Among these patients, 139 tested positives for the dengue NS1 antigen (Verma et al., 2022).

In 2022 outbreak in Nepal, the age profile of suspected dengue cases ranged from 2 to 91 years (median: 30 years, IQR: 22–42). Adults constituted 93.8% of cases, with 6.2% being children. Gender distribution was similar between dengue and non-dengue patients, but notable differences existed in age groups (Rimal et al., 2023). Patients with probable dengue ranged in age from 4 months to 76 years (median 27 years). A study conducted at STIDH in Kathmandu revealed that the median (IQR) age among dengue patients was 28 (22–39) years, and the male-to-female ratio stood at 1.2:1, showing no significant disparity between genders and dengue occurrences (Poudyal et al., 2021). In 2017, the ages 16–30 accounted for the largest proportion of dengue cases (49, 34.8%), followed by <15 years (32, 22.7%) (Tun et al., 2021). The median age of dengue patients was 29.5 years, with a higher prevalence observed in males, exhibiting a male-to-female ratio of 1.78. The patients' ages ranged from 12 to 74 years (mean age 31.18 ± 13.61 years), with the majority falling in the 16-45 age range. These findings suggest that the medical condition may be

more prevalent in males and in younger individuals (Prajapati et al., 2020). Additionally, 2017 study depicted that dengue was more prevalent among adults (86.8%) compared to children up to 15 years, with a child-to-adult ratio of 1:6.6 (Dumre et al., 2017). In the 2016 study, a higher occurrence of dengue infection was observed among males aged 19 to 41 years, with a child-to-adult ratio of 0.3:1 and a male-to-female ratio of 6:4. The average age of the patients was 37.85 years, with a standard deviation of 7.14 years (Khetan et al., 2018). In 2013, the analysis of 198 patients revealed a predominance of males aged 15-50 years, with a child-adult ratio of 0.2:1 (33/165) and a male-to-female ratio of 7:4, while the mean age (± standard deviation) was 45.75 ± 38.61 years, ranging from 2 to 77 years (Gupta et al., 2015). The rate of anti-Dengue IgM positivity was 10.7% in the age group above 50 years, 10.1% among individuals aged 15-50 years, and 8.8% in those below 15 years, with an equal male-to-female ratio of 1:1 (Shah et al., 2012).

In a study conducted in Pakistan, hematological findings revealed thrombocytopenia (platelet count ≤ 150,000) in 89% of cases, with 68% of patients exhibiting a hematocrit level below 40%, 22% between 41–45%, and 10% greater than 45% (Rahim et al., 2021). Hematological examination revealed thrombocytopenia in 69% of patients, with platelet counts and transfusion patterns being recorded daily to investigate the potential correlation between dengue severity and declining platelet levels (Verma et al., 2022). Patients who were admitted to the hospital showed clear differences in their hematological and biochemical profiles compared to those in the outpatient group during their first hospital visit. Notably, admitted patients had marked increases in lymphocyte count and significant decreases in platelet count (Rimal et al., 2023). Platelet counts were found to be below the normal range in 27.6% (24/87) of the patients, while 72.4% (63/87) of the patients (n = 87) had counts > 150,000/cm3. Both the total WBC count and hemoglobin level (Tun et al., 2021). A study conducted in Kathmandu found that the study population had normal hematocrit and hemoglobin levels, but platelet counts were below the normal range, with a median of 141,000 per cumm (Poudyal et al., 2021). Thrombocytopenia and leucopenia were notably prevalent among dengue patients in contrast to non-dengue cases (Dumre et al., 2017). During the 2013 study, thrombocytopenia, characterized by a platelet count below 100,000 per milliliter of blood, was observed in 147 individuals, accounting for 74.3% of the total study sample (Gupta et al., 2015).

3. Materials and methods

3.1 Study area

A cross-sectional molecular surveillance study for dengue was conducted in Jhapa district, Nepal. Jhapa, situated in the fertile Terai plains, holds the distinction of being the easternmost district in Nepal and falls within the Outer Terai region. The district shares its borders with Ilam to the north, Morang to the west, the Indian state of Bihar to the south, and the Indian state of West Bengal to the southeast and east. It covers an area of 1,606 square kilometers (620 square miles), with geographical coordinates ranging from 87°39' east to 88°12' east longitude and 26°20' north to 26°50' north latitude. Kankai Hospital, Birtamod; Birtamod Nagar Hospital, Birtamod; Shanischare Primary Hospital, Arjhundhara; and Pradeshik Hospital, Bhadrapur were identified as the centers for collection of biological samples in the 2023 outbreak and samples were collected in the month of September. Two hospitals in the outbreak region B & C Medical College Teaching Hospital & Research Centre, Birtamod, and Pradeshik Hospital, Bhadrapur were chosen to collect samples in 2022 from October to November.

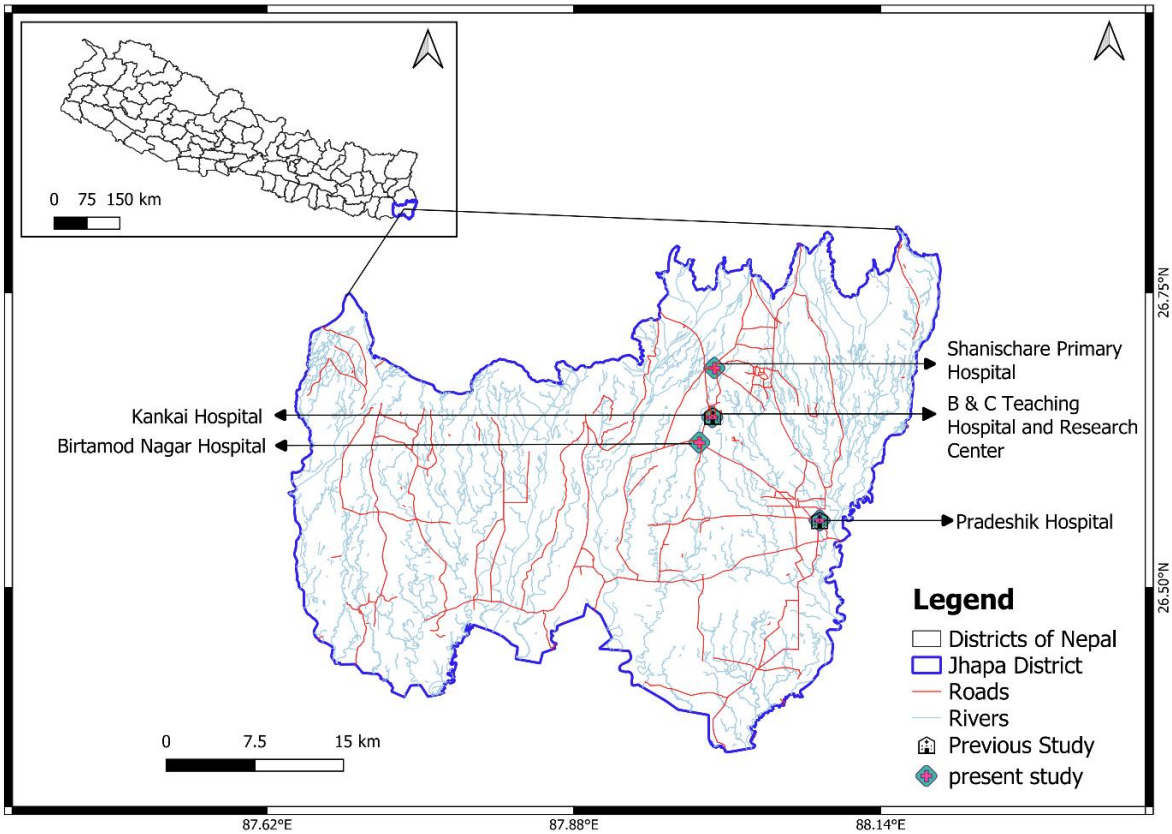

Figure 3. Map-depicting sample collection sites from Jhapa

3.2 Ethical approval

The Institutional Review Board approved the study (IRC/IOST 64/079/080) and written informed consent was obtained from all participants. The researchers and institutions engaged in the study ensure the anonymity of patients and uphold the confidentiality of data. Additionally, no individual-specific information is presented in this research.

3.3 Patient selection

The serological part of the study was designed as a descriptive cross-sectional study. Patients who tested positive for dengue were enrolled in this study. All participants underwent a comprehensive medical history review and a thorough physical examination upon admission. The samples belonged to the dengue outbreak of 2022 and 2023, collected between September-October. Upon testing positive by serology at respective hospitals, the samples were then stored in the deep freeze of the Red Cross society in Jhapa. They were transported in an icebox to the Central Department of Zoology Laboratory freezer and stored at -20°C for further molecular analysis.

3.4 Viral RNA extraction

A conventional reverse transcriptase-polymerase chain reaction (RT-PCR) was used to detect dengue genomic RNA (Lanciotti et al., 1992). Total RNA was isolated from the collected serum samples using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) following manufacturer instructions.

3.4.1 Procedure

The method involved pipetting 560 µl of prepared Buffer AVL containing carrier RNA into a 1.5 ml microcentrifuge tube. Subsequently, 140 µl of serum was added to the Buffer AVL–carrier RNA in the microcentrifuge tube. The contents were mixed by pulse-vertexing for 15 s and then incubated at room temperature for 10 min. Next, 560 µl of ethanol (96–100%) was added to the sample and mixed by pulse-vertexing for 15 s. Carefully, 630 µl of the solution was applied to the QIAamp Mini column in a 1.5 ml collection tube without wetting the rim. The cap was closed, and the tube was centrifuged at 8000 rpm. The step was repeated until all of the lysate was loaded onto the spin column. Afterward, 500 µl of Buffer AW1 was carefully added and the tube was centrifuged at 8000 rpm for 1 min. The QIAamp Mini column was then placed in a clean 1.5 ml collection tube, and the tube containing the filtrate was discarded. Carefully, the QIAamp Mini column was opened, and 500 µl of Buffer AW2 was added. The tube was centrifuged at full speed

14,000 rpm for 3 min. Continuing with the process, the QIAamp Mini column was placed in a new 1.5 ml collection tube, and the old collection tube with the filtrate was discarded. It was then centrifuged at full speed for 1 min at 8000 rpm. Subsequently, the QIAamp Mini column was placed in a clean 1.5 ml microcentrifuge tube, and the old collection tube containing the filtrate was discarded. Finally, 50 µl of Buffer AVE was carefully added to the open QIAamp Mini column. It was incubated at room temperature for 5 min and then centrifuged at 8000 rpm for 1 min. The viral RNA was extracted and stored at -20°C for immediate use in RT-PCR.

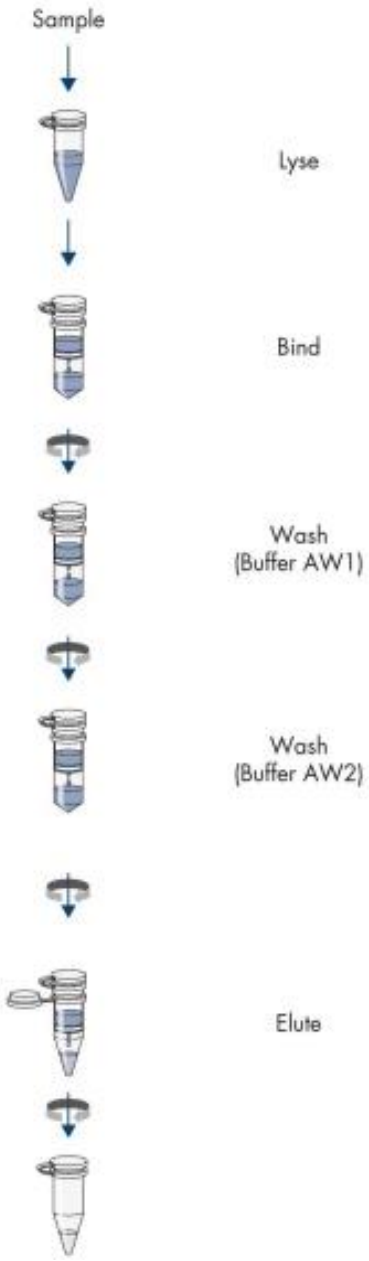

Figure 4. QIAamp Viral RNA Mini Spin pure viral RNA extraction procedure (Qiagen, 2020)

RT-PCR of DEN virus RNA was carried out with DENV consensus and serotype specific primers. Dengue RNA was reverse-transcribed into cDNA. DENV RNA presence was identified through RT-PCR using the Prime Script™ One Step RT-PCR Kit Ver. 2 (Takara Bio Inc., Shiga, Japan) in accordance with the manufacturer's guidelines. In brief, the RT-PCR amplification was conducted in a final volume of 12.5 µL with 2.5 µL of RNA (Poudyal et al., 2021).

Table 1. Preparation of maser mix for amplification

Components	**Amount**
Prime script enzyme	0.5µl
1 step buffer	6.5µl
Forward Primer	0.5µl
Reverse Primer	0.5µl
Nuclease free water	2µl
RNA template	2.5µl

The RT-PCR mixture included 0.5 µL of enzyme mix, 6.5 µL of 2× buffer, 2 µL of nuclease-free water, and 0.5 µL of 100 pmol forward and reverse primers. Separate primer sets were used for DENV detection and identification of specific DENV serotypes (Poudyal et al., 2021).

Table 2. List of primers used in RT-PCR for the detection and serotyping of dengue virus

Dengue type	Target region	Primer sequences	Amplicon size(bp)
Dengue consensus	C and prM	5'-TCAATATGCTGAAACGCGCGAGAAACCG-3' 5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3'	511
Dengue 1	E and NS1	5'-GGACTGCGTATGGAGTTTG-3' 5'-ATGGGTTGTGGCCTAATCAT-3'	490
Dengue 2	E and E	5'-GGGTCTTGAGACATCCAGGC-3' 5'-TCTTCCCCTGAGTGAGGTGT-3'	565
Dengue 3	E and NS1	5'-GTGCTTACACAGCCCTATTT-3' 5'-TCCATTCTCCCCAAGCGCCTG-3'	320
Dengue 4	NS2a and NS2b	5'-CCATTATGGCTGTGTTGTTT-3' 5'CTTCATCCTGCTTCACTTCT-3'	399

PCR amplification was performed using MyGene™ Series Peltier Thermal Cycler (Hangzhou LongGene ® Scientific Instruments Co., Ltd., Zhejiang, China) (Table 3). To identify the DENV serotype, the same PCR recipe and conditions as in the dengue consensus reaction above were performed employing DENV serotype specific primer sets. For every cycle, both negative and positive controls were incorporated.

Table 3. Thermal profile for amplification

Stage	Program	Temperature(°C)	Time
Stage I	Initiation	50	30 min
Stage II	Initial Denaturation	94	2 min
Stage III	Denaturation	94	30 sec
35 cycles	Annealing	54	30 sec
	Elongation	72	1 min
Stage IV	Final Elongation	72	7 min
Stage V	Holding	10	Infinity

3.5 Gel electrophoresis

3.5.1 Preparation of agarose gel matrix

The gel was prepared by dissolving 0.6g of agarose powder in a 30 ml 1×TBE buffer, and it was boiled in the microwave for 1–3 minutes until all the crystals of agarose were completely dissolved. The concentration of agarose in a gel is 2%. The solution is then cooled to approximately 55°C. 4µl of ethidium bromide was added to the solution and stirred again. A comb was placed across the end of the casting tray to form wells when the gel solution solidified. After the gel solidified, it was submerged in a buffer-filled electrophoresis chamber that contained a positive electrode (anode) at one end and a negative electrode (cathode) at the other.

3.5.2 Sample preparation and loading

Samples were prepared for electrophoresis by mixing them with 6×loading dyes. The 5µl of PCR reactions were mixed with 1µl of loading buffer. Then it was mixed well by gently pipetting up and down several times until the color of the liquid was homogenous. The samples were added to the gel well, and the DNA ladder was loaded in the first well. The lid was placed on the gel box. The power supply was turned on at about 120 volts for about 30 minutes. Visualization was performed using the UVITEC Gel Documentation system. Thorough precautions were exercised during RNA extraction and RT-PCR processes to mitigate the risk of cross-contamination among samples.

3.6 Data analysis

Demographic and hematological data were entered into a Microsoft Excel spreadsheet. Descriptive analysis was used to summarize the characteristics of the participants. Statistical analysis was done using a R Core Team (2023) version 4.3.2 (R Core Team & R Foundation for Statistical Computing, 2023). Group differences were compared using the Pearson Chi squared test or Fisher's exact test for categorical variables, or the student t test or the Mann–Whitney U test for continuous variables and expressed as mean and standard deviation (SD). A difference was considered to be statistically significant at a p-value of less than 0.05.

4. Results

4.1 Serotype circulation and distribution of dengue virus

In 2022, among the study population, 150 individuals (51.7%) were female (male to female ratio of 1:1.07), with a median age of 33 years. Age of one patient was missing in the data. The age group with the highest incidence of dengue-positive cases was individuals aged 15-45 years, representing 40.8% of all suspected cases, while the lowest occurrence was observed in individuals below 15 years old, constituting 34.6% of total cases. There were no significant differences in gender between dengue and non-dengue patients ($p > 0.68$), although a significant difference was noted in age groups ($p < 0.05$) (Table 4). Out of the 46 positive samples, 25 tested positives for dengue IgM antibodies, and 28 were positive for NS1 antigen. Additionally, nine samples tested positive for both NS1 and IgM antibodies (Table 5).

Table 4. Distribution of demographic variables for dengue suspected patients in Jhapa District, Nepal 2022.

Variables	Dengue Positive Patients (%)	*p* Value
Gender (n = 290)		
Male	22 (7.58)	0.68
Female	24 (8.27)	
Age (n = 290)		
<15	6 (2.07)	**<0.05**
15-45	27 (9.34)	
>45	13 (4.49)	

In 2023, among the 66 subjects, 37 (56%) were male (female to male ratio of 1:1.28), with a median age of 33 years. The age of the study subjects ranged from 1 to 94 years with a mean age of 34.6 years. The age and gender distribution of dengue positive cases included in this study is shown in (Figure 5a). The majority of the study subjects were 15-45 (54% of total study population).

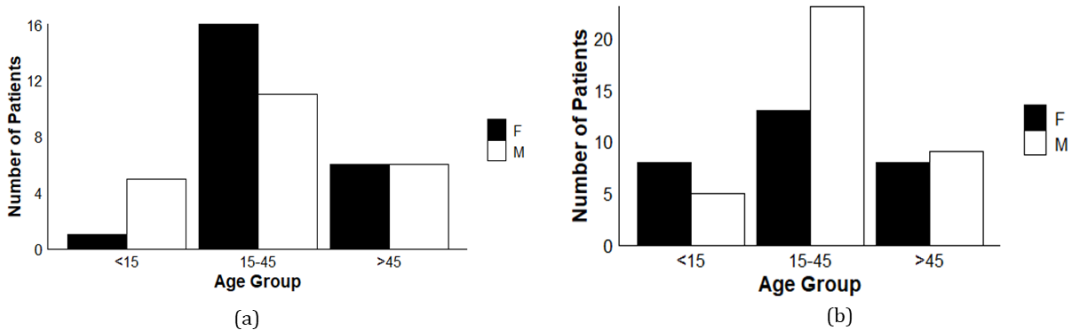

Figure 5. Age and gender wise distribution of DENV-infected patients in 2022 (a) and 2023 (b)

Out of 64 samples, 76.6% (n=49) tested positive for NS1 only, while 15.6% (n=10) tested positive for IgM only. Furthermore, 7.8% (n=5) of the samples tested positive for NS1 and IgM. Out of 60 samples examined, 44 tested positives for Dengue via RT-PCR. Upon comparing these 60 samples, it was noted that 4 individuals who were NS1 seropositive tested negative by RT-PCR, along with 8 individuals who were IgM seropositive and 2 individuals who were positive for both NS1 and IgM but tested negative in RT-PCR.

Table 5. Dengue NS1 and IgM positive cases in 2022 and 2023 detected through RDT test.

Test	**2022**		**2023**	
	IgM +	**IgM -**	**IgM +**	**IgM -**
NS1 +	9	28	5	49
NS1 -	16	286	10	2

The DENV serotypes identified were DENV-2 (31), DENV-3 (13) and DENV-1 (2); DENV- 2 was found to be the major serotype identified with 67.4% of the test subjects followed by DENV-3 with 28.3 % (Table 5). There is a significant difference in distribution of dengue serotype ($p<0.001$) (Table 6). The RT-PCR examination validated the presence of three dengue serotypes specifically, DENV 1-3 while Dengue serotype 4 was not observed in any of the samples.

Table 6. Distribution of dengue serotypes.

S. N.	Serotype	Number (%)	X^2	*p* value
1	DENV-2	31(67.4)		
2	DENV-3	13(28.3)	30.045	**<0 .001**
3	DENV-1	2(4.3)		

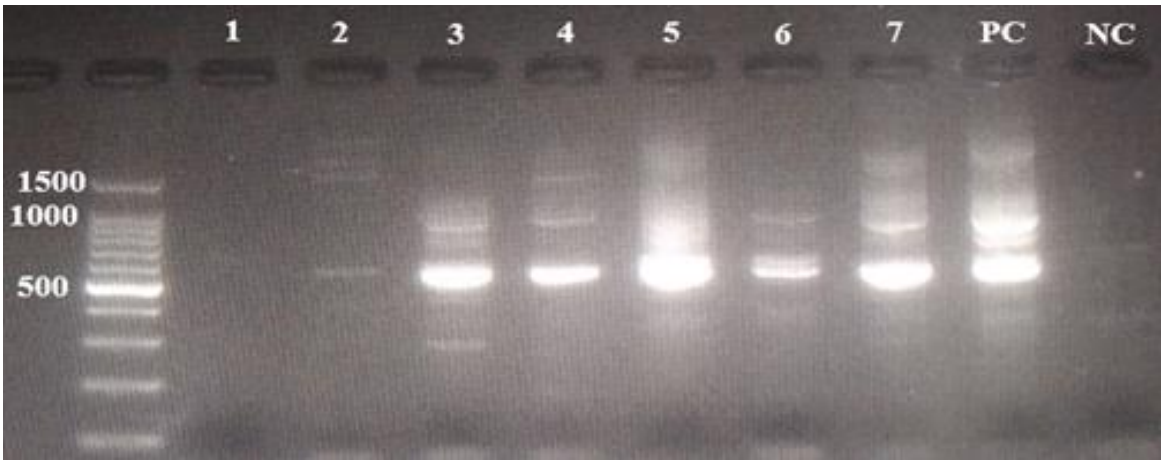

Figure 6. PCR product of DENV 2 serotype (565 bp) under UV illuminator. 1st well = Molecular weight marker (100 bp); 1,2,3,4,5,6,7, = Samples; PC = Positive control; NC = Negative control

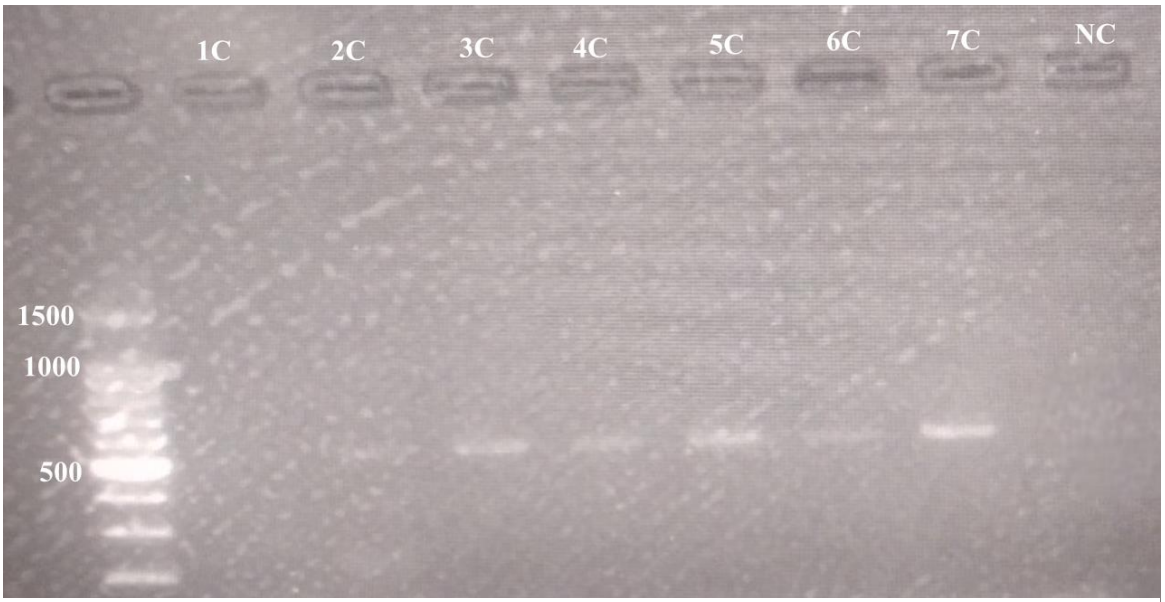

Figure 7. PCR product of DENV consensus (511 bp) under UV illuminator. 1st well = Molecular weight marker (100 bp); 1C,2C,3C,4C,5C,6C,7C, = Samples; NC = Negative control

Out of the chosen 66 subjects, 37 (56%) were male, with a median age of 33 years. The age of the study subjects ranged from 1-94 with a mean age of 34.6. The age and gender distribution of positive dengue cases included in this study is shown in (Figure 5b). The majority of the study subjects were 15-45 (54% of total study population).

Out of 64 samples, 76.6% (n=49) tested positive for NS1 only, while 15.6% (n=10) tested positive for IgM only. Additionally, 7.8% (n=5) of the samples tested positive for both NS1 and IgM. Out of 60 samples examined, 46 tested positives for Dengue via RT-PCR. Upon comparing these 60 samples, it was noted that 6 individuals who were NS1 seropositive tested negative by RT-PCR, along with 10 individuals who were IgM seropositive and 2 individuals who were positive for both NS1 and IgM but tested negative in RT-PCR. Dengue NS1 antigen was not detected in 2.17% (1/46) of PCR positive samples but was detected in 42.85% (6/14) of PCR negative samples.

Table 7. Distribution of demographic variables for RT-PCR confirmed dengue patients in Jhapa District, Nepal 2023

Variables	**n**	**DENV Positive Patients (%)**	***p* Value**
Gender			
Male	36	27 (45)	0.95
Female	24	19 (31.67)	
Age			
Below 15	9	6 (10)	0.70
15-45	36	27 (45)	
Above 45	16	13 (43.33)	
NS1 Antigen Detection			
Positive	50	44 (73.33)	**<0.001**
Negative	9	1 (1.67)	

4.2 Hematological parameters and dengue virus

The study identified a significant association between Dengue infection and leukocyte count, with 40% of individuals who tested positive for Dengue having leukocyte counts below 4000 mm³ ($p<0.001$). There was no statistically significant variance observed in the platelet count, hemoglobin, and hematocrit levels between patients who tested positive and negative for Dengue virus infection ($p>0.05$) (Table 8).

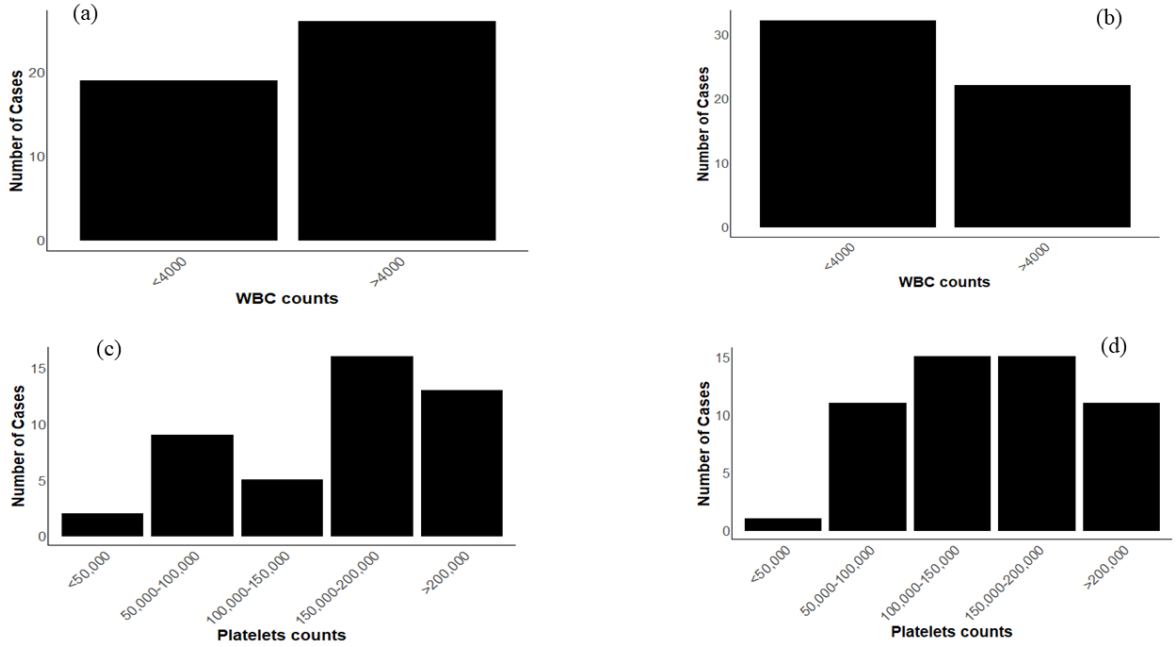

Figure 8: Haematological parameters in dengue RDT-positive patients: WBC counts in 2022 (a) and in the 2023 outbreak (b), platelets count during the 2022 outbreak (c), and in the 2023 outbreak (d) in Jhapa District, Nepal

Table 8. Comparison of haematological parameters of dengue positive and negative patients in 2022

Hematological Parameters	**Dengue positive patients (mean ± sd)**	**Dengue negative patients (mean ± sd)**	***p* value**
WBC	6153.3 ± 3131.1	8405.6 ± 3536	**<0.001**
Platelets	170863.6 ± 84036.4	189497.9 ± 154591.2	0.25
Hemoglobin	12.1 ± 1.9	11.7 ± 1.6	0.16
Hematocrit	34.9 ± 5.5	33.9 ± 5	0.26

The majority of patients (28, or 48.3%) had thrombocytopenia (platelet count <150,000), with only 1 patient (0.2%) having severe thrombocytopenia (platelet count <50,000) (Figure 7). No differences were found between the positive cases in terms of age, gender, NS1 antigen detection, platelets, leukocytes, hemoglobin, or hematocrit value at presentation ($p > 0.05$) (Table 9).

Table 9. Comparison of haematological parameters of dengue positive and negative patients in 2023

Hematology Parameter	**Dengue positive patients (mean ± sd)**	**Dengue negative patients (mean ± sd)**	***p* value**
WBC	4543.9 ± 2056.1	5746.1 ± 4158.1	0.9
Platelets	148625± 55020.6	168461± 88685.4	0.7
Hemoglobin	12.5 ± 1.6	12.7 ± 1.8	0.67
Hematocrit	39.7 ± 4.1	37.6 ± 4.9	0.2

5. Discussion

Globally, dengue presents a major challenge to public health. Dengue's incidence varies considerably across countries, but its burden is increasing globally. In the past two decades, dengue incidence has increased dramatically, posing a substantial public health challenge. More than five million cases of dengue have been reported since the beginning of 2023 due to ongoing transmission and an unexpected spike in the number of cases. Over 5000 deaths have been reported in over 80 countries/territories and five WHO regions: Africa, Americas, South East Asia, Western Pacific, and Eastern Mediterranean (WHO, 2023).

In June 2023, an outbreak of the dengue virus commenced in Sunsari district, situated approximately 79 kilometers away from Jhapa district, serving as the epicenter of the outbreak and resulting in a total of 667 reported cases during that month (EDCD, 2023). Dengue is endemic in Jhapa, as in other districts in Nepal. Jhapa has also experienced significant impact, recording approximately 3,800 cases this year, marking a record high (EDCD, 2023). Although dengue is endemic, there haven't been any documented dengue virological data from Jhapa as far as we know. As a result, this research offers the first details on the serological profile, distribution of serotypes, genetic diversity of the virus, and sources of the DENV that is circulating locally in jhapa district.

In 2022, a sample comprising 290 suspected cases was collected due to the high number of dengue suspected patients seen in hospitals, although the prevalence rate was only 15.9%. In 2023, a total of 66 samples tested positive on RDTs were collected due to a significant outbreak. Serotype examination of 2023 was focused as serotype distribution of 2022 outbreak in Nepal was published (Rimal et al., 2023). According to the age distribution of dengue cases, the age group between 15 and 45 years old accounted for the largest percentage of cases (54.5%), with individuals over 45 years old coming in second (25.7%). According to this study, age has a statistically significant impact on the likelihood of infection ($p<0.05$). This outcome is consistent with data from the previous outbreak in Nepal, when higher cases of dengue were reported among individuals over the age of 15 (Gupta et al., 2015; Rimal et al., 2023; Tun et al., 2021a). Ages under 15 are considered paediatric, those between 15 and 45 are considered medium or active, and those over 45 are considered old. Due to their increasing outside activity, people in their middle years are more likely to come into touch with vectors. This age group has greater economic

significance. Because of this, there is a greater likelihood of hospitalization in this age range than in others.

In 2022, among 46 RDT positive cases, 22(47.82%) were males and 24 (52.17%) were females with male to female ratio of 1:1.09. Statistically, there was no significant relation between sex and the occurrence of the disease ($p>0.05$). The result was in accordance to the previous studies in which the number of DV cases was more in females (Ridde et al., 2016). In 2023, among 46 RT-PCR positive cases, 27 (58.69%) were males and 19 (41.30%) were females with male to female ratio of 1.42:1. Statistically, there was no significant relation between gender and the occurrence of the disease ($p>0.05$). The result was in accordance to the previous studies in which the number of DV cases was more in males 1.5:1 (Pun 2011; Shah et al., 2012). The numbers of male cases were higher than the female that might be due to their greater involvement in outdoor activities.

In primary infections, NS1 can be identified concurrently with viral RNA and prior to the development of an antibody response (Muller et al., 2017). Therefore, 88% of NS1 positive samples had accurate RT-PCR detection. Six samples that were initially determined to be NS1-positive did not yield positive RT-PCR test findings. Poor temperature control during sample transportation may have caused false negatives in the RT-PCR data. Sample collection, preservation, and transfer must be handled securely to ensure the stability and quality of analyzed samples, resulting in more accurate and reliable results.

This study found that 67% of the dengue serum samples had serotype 2. According to a 2019 study conducted in Kathmandu, DENV-2 was the most prevalent serotype in 63.1% of the serum samples. Other investigations revealed analogous predominance for this serotype (Yung et al., 2015). Between 1990 and 2015, India had the greatest number of outbreaks globally, followed by China and Brazil (Guo et al., 2017). The identification of all four DENV serotypes in India between 2019 and 2021, particularly with the dominance of DENV-2, suggests a scenario that could potentially influence the circulation of the same serotype in the current outbreak in Nepal (Agarwal et al., 2023). The five isolates of DENV-2 from Indian strains were obtained from the Terai region, situated along the border of India (Tun et al., 2021). Nepal's extensive and unrestricted border with India, coupled with frequent travel by Nepalese individuals to India for employment and business, underscores a notable migration dynamic. The absence of visa requirements facilitates substantial movement of people between the two nations. During the 2023 outbreak, it is plausible that

returning migrant workers, previously infected with the dengue virus in India, served as sources for local transmission in Nepal through mosquito vectors. Furthermore, the concurrent occurrence of DENV-2 outbreaks in Nepal, India, and Pakistan suggests a regional correlation, indicating that the DENV-2 outbreak in Nepal likely originated from viral strains in India. However, it is imperative to acknowledge that the available information from India remains limited, underscoring the need for comprehensive cross-border collaboration in dengue research. The increasing movement of people between Nepal and India may allow a more frequent introduction of the dengue viruses in Nepal.

Patients who tested positive for dengue in 2022 showed a drop in white blood cell (WBC) counts. This observation is consistent with results from other research that have yielded comparable conclusions. This study has a limited sample size, which may affect the reliability of the analysis. As a consequence, a definitive conclusion can't be drawn regarding the association between DENV infection and demography and hematology. According to the research findings, severe dengue exhibited a sevenfold higher frequency in DENV-2 cases compared to other serotypes (Vicente et al., 2016). This observation contributes to the ongoing discourse on the differential impact of Dengue virus serotypes on disease severity. A noteworthy observation in the study was the involvement of DENV-2 in this outbreak, indicating a serotype change from the previous 2022 outbreak, which was attributed to DENV-1. These alterations in the circulating dengue virus serotype increase the population's susceptibility to severe infections due to antibody-dependent enhancement (ADE) in conjunction with the inherent virulence of the infecting strain (Teo et al., 2023).

6. Conclusion and recommendations

6.1 Conclusion

This study provides the first molecular and virological characteristics of DENV in the Jhapa district of Nepal. The extensive outbreak in Jhapa, Nepal in 2023 revealed the coexistence of three distinct DENV serotypes, with DENV-2 and DENV-3 prevailing as the most prominent. This observation implies occurrences of serotype displacement, highlighting the notable resurgence of DENV-2 following a three-year period, alongside significant viral loads. These findings are important in planning for future epidemics. Continual molecular and virological surveillance of DENV will help better understand dengue disease dynamics in this region and contribute to disease prevention and management efforts.

6.2 Recommendations

- Since early detection of circulating serotypes may be an effective strategy to anticipate the number of severe outcomes during dengue outbreaks, serotype circulation surveillance should be conducted continuously and extensively.
- Develop targeted prevention and control strategies based on prevalent serotypes like DENV-2 and DENV-3.
- Foster collaboration for further research on dengue epidemiology and vector dynamics.

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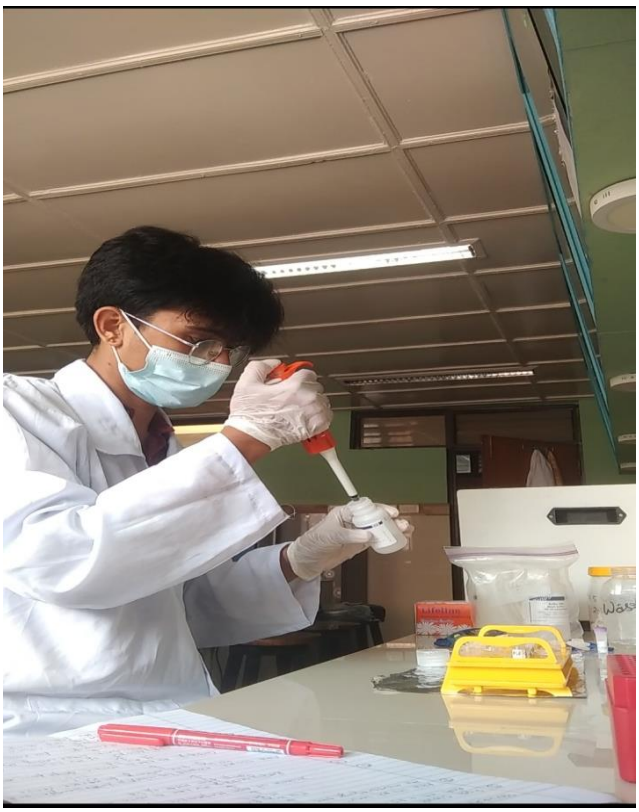
Appendices

Appendix 1. Photographs

Photograph 1: Serum sample collection

Photograph 2: RDT test of dengue suspected serum

Photograph 3: Transport of serum to CDZ lab



Photograph 4: Dengue viral RNA extraction

Appendix 2. Ethical approval letter

Tribhuvan University
Institute of Science and Technology
Kirtipur, Kathmandu, Nepal

Institutional Review Committee

IRC/IoST Chairperson
Assoc. Prof. Dr. Surendra Gautam
Asst. Dean-Academics, IoST

IRC/IoST Members
Prof. Dr. Rajani Malla
Prof. Dr. Sangeeta Rajbhandary
Prof. Dr Shankar P Khanal
Prof. Dr Kumar Sapkota
Prof. Dr. Amar Prasad Yadav
Prof. Dr. Prakash Ghimire
Assoc. Prof. Dr. Megha R Banjara
Assoc. Prof. Dr. Nirmal Kumar Raut
Dr. Supriya Sharma

Member Secretary
Assoc. Prof. Dr. Komal Raj Rijal
Head, Central Department of Microbiology

IRC/IOST Secretariat
Central Department of Microbiology
Phone: 4331869

Ref. No.: IRC/IOST 64/079/080 Date: 4 March, 2023

PI: **Dr. Kishor Pandey**
M.Sc student: **Niten Bharati**
Central Department of Zoology, TU
Kirtipur

Ref.: IRC Ethical Approval of research proposal entitled **"Molecular detection of dengue virus outbreak in Jhapa district, Nepal"**

Dear**Dr. Pandey**

It is our pleasure to inform you that the above mentioned proposal submitted on 22 November, 2022 (Regd. No **IRCIOST-23-0005**), following independent expert review and discussion in the IRC/IoST meeting held on **03 March, 2023** has been approved for implementation [start date 04 March, 2023 and end date 03 March, 2024], maintaining ethical principles, set by the Nepal Health Research Council.

The investigators have to strictly follow the protocol stipulated in the proposal. Any change in objective(s), problem statement, research question or hypothesis, methodology, implementation procedure including deviation of the protocol, data management and budget need to be submitted in detail with justification for seeking prior approval to implement the proposed change including extension of the date, in the protocol.

Further, the researchers are also directed to follow the national ethical guidelines published by Nepal Health Research Council during the implementation of research. You are required to submit the final report to the IRC within a month of completion of the research, as planned in the approved proposal.

If you have any questions, please contact the Institutional Review Committee of Institute of Science and Technology, Tribhuvan University.

Thanking you,

.....
Assoc. Prof. Dr. Komal Raj Rijal
Member Secretary
Institutional Review Committee
Institute of Science and Technology
Tribhuvan University