Identification of conserved peptide containing HLA restricted epitopes of *Puumala orthohantavirus* nucleocapsid protein

A Dissertation

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Certificate

This is to certify that dissertation titled "Identification of conserved peptide containing HLA restricted epitopes of *Puumala orthohantavirus* nucleocapsid protein" submitted by Ms. Ayushi Sehgal in partial fulfilment of the requirements for the award of the degree of Master of Technology in Biotechnology, Thapar Institute of Engineering and Technology, Patiala, is a bonafide work carried out by her under my supervision and guidance.

To the best of my knowledge, the dissertation work contains no materials previously submitted to any other university or institute for the purpose of receiving a degree or certificate.

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Declaration

I hereby declare that the work submitted under the dissertation titled "Identification of conserved peptide containing HLA restricted epitopes of *Puumala orthohantavirus* nucleocapsid protein" is an original record of my research work which was carried out under the supervision of Dr. Manoj Baranwal, Professor at Thapar Institute of Engineering and Technology, Patiala. Previously no other institution or University has ever submitted this research work as a project or thesis for the purpose of receiving degree or diploma.

Place: Patiala

Date: **12/09/2022**

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Abstract

Puumala orthohantavirus is a re-emerging zoonotic virus distributed primarily in Europe and Russia that causes nephropathia epidemica, a mild form of Hemorrhagic fever with renal syndrome (HFRS) that infects thousands of individuals annually. The demand for a vaccine against the Orthohantavirus is on the rise due to the lack of enduring treatment and vaccination. This study focuses on design of multiepitope peptides as a vaccine candidate against *Puumala orthohantavirus* using various immunoinformatic approach. Five conserved (90% conservancy) peptides of nucleocapsid protein of PUUV were identified. Three conserved peptides containing multiple T and B cell epitopes were selected with the usage of different computational epitope prediction tools. Molecular docking (HPEP dock server) of epitopes of selected reveal good binding interaction with HLA alleles (ten alleles for each class I and II HLA). IEDB population coverage analysis showed more than 90 % average population coverage of identified peptides across six continental regions (Asia, Africa, Europe, North America, South America and Australia). Collectively, these immunoinformatics analysis support the immunogenic potential of these peptides which may be considered as vaccine candidate against PUUV.

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List of abbreviations

AKI	Acute Kidney infection
ANDV	Andes orthohantavirus
CDR	Coding regions
DOBV	Dobrava-Belgrade virus
GPC	Glycoprotein precursor
HFRS	Haemorrhagic fever with renal syndrome
HCPS	Hantavirus cardiopulmonary syndrome
HTNV	Hantaan orthohantvirus
NCR	Non coding regions
N.E	Nephropathia epidemica
PUUV	Puumala orthohantavirus
SEOV	Seoul orthohantavirus
SNV	Sinnombre orthohantavirus
THAIV	Thailand orthohantavirus
TMPV	Thottopalayam orthohantavirus
TULV	Tula orthohantavirus

Chapter1: Introduction

Puumala is a zoonotic virus, a member of the hantaviridae family, genus Orthohantavirus [1]. In 1979, *Puumala orthohantavirus* (PUUV) was first encountered in Finland from *Myodes glareolus* (bank voles), which belongs to the *Arvicolinae* subfamily of the *Cricetidae* family [2,3]. It is responsible for developing an infection in both rodents and humans. In rodents, it causes an asymptomatic and chronic disease, while, In humans, it causes a mild form of hemorrhagic fever associated with renal syndrome (HFRS) with a fatality rate of around 0.4 % which is also known as the nephropathia epidemica (N.E) [4,5]. N.E is manifested by thrombocytopenia, high fever, cephalalgia and acute kidney injury(AKI)[6]. Having severe AKI is the most common reason for death in HFRS patients. Incubation period of HFRS generally ranges between 2 to 4 weeks. HFRS clinically progresses through five phases: febrile, hypotensive, oliguric, polyuric and convalescent. These five phases may be well-demarcated in severe HFRS but may overlap or be absent in mild and moderate cases [7,8].

A majority of PUUV infection cases clinically present as nephropathia epidemica are endemic in Europe and western Russia [9,10], with approximately 10,000 reported cases each year [11]. Transmission of infection in humans may occur for several reasons, a) rodent bites, b) contact with rodent's urine, droppings or saliva, and c) ingesting food contaminated with urine, droplets, or saliva from infected rats[12].

Puumala Orthohantavirus, a single-stranded, negative-sense enveloped RNA virus, having a trisegmented genome(tripartite) containing three segments: small (S), medium (M) and large (L). These RNAs encode nucleocapsid (N) protein, glycoprotein precursor (G1 and G2), and RNA-dependent RNA polymerase (RdRp) protein, respectively[1]. Several researchers has reported in their study that glycoprotein and nucleoprotein of Orthohantavirus elicits the humoral immune response [105–107]. Nucleoprotein(N) protein is most abundant protein and plays multiple functions; it activates non-neutralizing antibody [109], stimulate T cell immune response [110,111] and induces the cross reacting immune response [112,113]

The location of the natural host reservoir determines the geographic distribution of Orthohantavirus, as these viruses are establishing the chronic infection in specific rodents (considered as natural host reservoirs) [7,45,170].The pathogenic behavior of Orthohantavirus also depends on their geographical location, so developing a strain-specific vaccine is necessary. Presently, eight different lineages of PUUV are found across different geographic regions of Europe and Asia (Eurasia)[5].

Currently, no specific treatment or preventive measures (vaccines) have been approved by the FDA [11]. Ribavirin and Favipiravir (antiviral drugs) are used as supportive treatment. However, these medications have serious detrimental effects, like hemolytic anaemia and are not suitable to consume during pregnancy. Vaccine failure may occur due to genetic diversity and the broad host range of orthohantavirus families [13]. Though three types of vaccines are against HFRS: killed Vaccine, DNA vaccine, and Live Attenuated Vaccine [14]. An example of a killed vaccine is the Hantax Vaccine, designed by the formulation of rodent brain using conjugation with adjuvant (aluminium hydroxide). This vaccine is authorised to use on humans in Korea and China only; Other nations stopped and deterred this approach because of the utilisation of rodent brains. The other two Vaccines are still in phases of preclinical trials [14,15].

Recent years have progressed in developing vaccines based on peptides using the immunoinformatic approach. In peptide-based vaccines, multiple immunogenic protein fragments are combined to make one fragment capable of triggering the immunogenic response[16]. This study investigated the conserved peptide fragments of nucleocapsid protein of *Puumala orthohantavirus.* Further, the conserved immunogenic peptides containing multiple epitopes of both B and T cells were selected with the use of different epitope prediction algorithms. Lastly, the peptide fragments were assessed for their binding interactions with HLA alleles by performing molecular docking and population coverage analyses.

Chapter 2: Review of Literature

2.1 Orthohantavirus

Orthohantaviruses, zoonotic pathogens, belong to a genus, *Hantavirus*, family, *Bunyaviridae* [17]. There are multiple viruses in the genus *Hantavirus*, many of which are human pathogens: Hantaan virus (HNTV), Seoul virus (SEOV), Sin Nombre virus (SNV), Puumala virus (PUUV), Andes virus(ANDV) and Dobrava-Belgrade virus (DOBV). More than Fifty Orthohantavirus species are presently known, out of which around twenty-four are pathogenic and capable of causing infectious diseases in humans [18]. The virus has been divided into two major groups: Old World and New World Hantavirus, which causes two acute febrile diseases: hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS)[17,19]. HFRS and HCPS cases have been confined to the geographic distributions of the natural small rodent host distribution [19]. The Old world group of orthohantaviruses: DOBV [20], HTNV [21] and PUUV [22], cause HFRS to circulate primarily in small rodent populations living in Europe and Asia and the new world group of orthohantaviruses such as SNV[23] and ANDV[24], causes HCPS mainly inhabits in North and South American countries.

2.2 *Puumala Orthohantavirus* (PUUV)

In 1979, PUUV was first encountered in *Myodes* glareolus (bank vole) from a Finnish municipality known as Puumala [25]. The *Myodes glareous* serves as a host reservoir for the *Puumala orthohantavirus*, which belongs to the Arvicolinae subfamily[2,3] (Figure1). Due to the geographical distribution of the host reservoir (bank vole), it is mainly found in Northern Europe and Russia and causes an infectious disease known as nephropathia epidemica (N.E) [26]. Nephropathia epidemica is a mild type of hemorrhagic fever associated with renal syndrome, manifested by cephalalgia, pyrexia, thrombocytopenia and acute kidney injury [6]. An outbreak occurs when the population of the host reservoir (bank vole) increases faster than expected concerning the environmental conditions, resulting in a higher rate of infection distribution among the rodents[27].

Figure 1: *Myodes glareous* (Bank vole) host reservoir of PUUV[28]

2.3 Genome of *Puumala orthohantavirus*

Puumala orthohantavirus is a negative-sense single-stranded, spherically enveloped RNA virus, having a diameter ranging from 80-120 nanometer (nm). PUUV has tripartite genome, which contains three segments: small segment (S), medium segment (M) and large segment (L), shown in figure 2 [29] . Also, these tri-segmented genome contains coding regions(CDR) and Non-coding regions(NCR), represented in figure 3.

The Small segment (S) is responsible for encoding two proteins, nucleocapsid(N) and a nonstructural protein(NSs)[29,30]. Nucleocapsid protein is an abundant protein consisting of 433 amino acid residues and 1828 bases of nucleotides. It plays several pivotal roles like intracellular transit as well as assembly of the viral genome. Furthermore, the N protein aids the virus's adhesion to host cell proteins and encourages replication[31]. Many studies have shown that nucleocapsid are highly conserved proteins across all Orthohantavirus species. They can be utilised to diagnose infection since their transcripts are expressed early in the infected cell's cytoplasm[32]. On the other hand, Nonstructural proteins(NSs) suppress the interferon-beta promoter (IFN-ß), which regulates the expression of interferons in infected cells.

The medium segment is responsible for encoding glycoproteins, which are further processed by post-translational modification at the cleavage site known as WAASA and result in two forms of glycoproteins, G1 and G2 [33]. It is made up of 1138 amino acid residues and 3650 nucleotide bases. The genome of hantavirus is enveloped within lipid bilayers, and the bilayer

helps to protect the hantavirus genome[29]. Glycoproteins are the surface spike proteins present on the bilayer and are responsible for promoting the attachment of the target cell using the integrin receptors[34].

The large segment(L) is mainly responsible for encoding RNA dependent RNA polymerase(RdRp). It is made up of 2155 amino acid residues and 6550 nucleotide base pairs. Transcription and replication are two major stages of the orthohantavirus life cycle, both processes are regulated by the RdRp protein[28]. Transcripts of the viral RNA are transcribed into the messenger RNA(mRNA) and then translated into proteins. Replication occurs via a cRNA intermediate and produces S, M and L viral RNA[35].

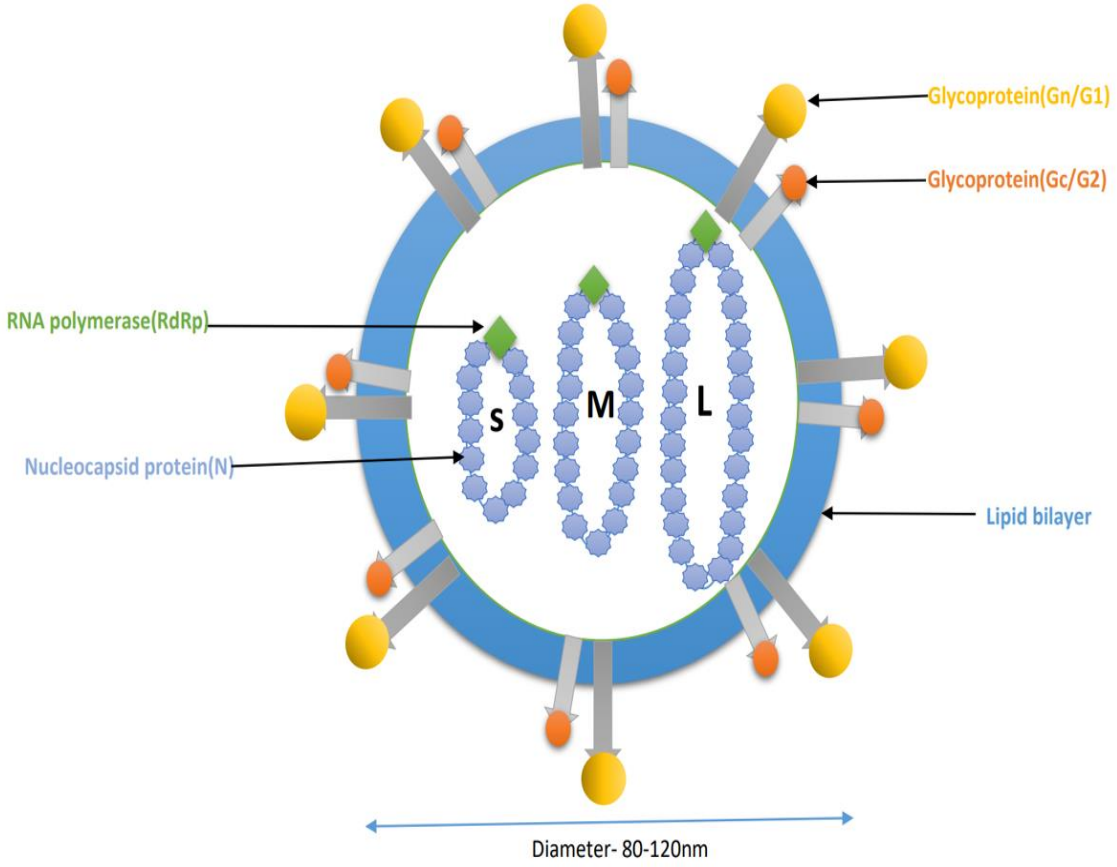

Figure 2: Schematic representation of *Puumala orthohantavirus* virion. The structure is spherical, having a diameter varying from 8-120nm, enveloped with a lipid bilayer containing spikes of glycoproteins (G1 and G2). Inside the virion, it includes three segments of single-stranded RNA Small(S), Medium(M) and Large(L), respectively.

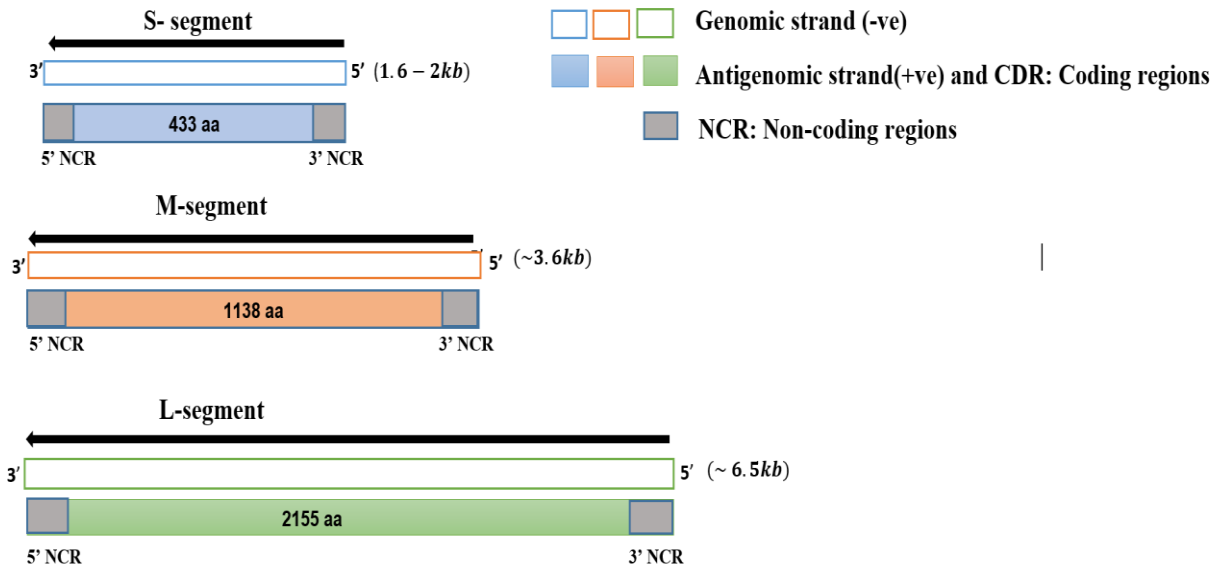

Figure 3: A linear representation of the *Puumala orthohantavirus* tripartite genome S, M, and L segments. S segment encodes for 433 aa of nucleocapsid protein, M segment encodes for 1138 aa of glycoprotein (Gn and Gc), and L segment encodes for 2155 aa of RNA dependent RNA polymerase. Coding and non-coding regions (CDR and NCR) are represented by different colours.

2.4 Transmission of Orthohantavirus

The virus transmission is the most crucial stage in establishing infection. The most common route of orthohantavirus transmission is via inhaling aerosol contaminated with virus released by rodents such as deer mouse (*Peromyscus maniculatus*), rice rat (*Oryzomys palustris*), white-footed mouse (*Peromyscus leucopus*) and the cotton rat (*Sigmodon hispidus*) [36]. It was demonstrated that orthohantaviruses could be transmitted by the intramuscular injection; however, the aerosol rout appears to have about 100 times higher efficacy in establishing infection. This data supports the notion that the orthohantaviruses spread primarily by inhaling aerosol contaminated with the virus. The virus containing aerosol could be produced when fresh rodent urine, droppings, or nesting materials are stirred up and small droplets containing the virus become released into the air.[37]. In addition to the aerosol inhaling, orthohantaviruses could be transmitted by: (a) rodent bites, which is rare, (b) contact with rodents' urine, dropping or saliva, (c) ingesting food contaminated with urine, droplets, or saliva from infected rats[12].

Although rodents are the main source of infection, orthohantaviruses have a constrained ability to spread among humans. As far as old-world Hantaviruses, no case of person-to-person transmission has been reported [38]. The only outbreak of person-to-person transmission were shown in Argentina due to Andes virus[39]. Each hantavirus may have a different strategy for transmission. There is one thing that Orthohantavirus transmission always has in common that it depends on its survival in the environment [40,41]. Studies have demonstrated that PUUV, an orthohantavirus circulating in bank voles, does not require direct contact between rodents and could be remain infectious for the long time on contaminated fomites [41].

2.5 Geographical distribution of Orthohantavirus lineages

Rodent species associated with orthohantaviruses are divided into two families: *Muridae* [42] and *Cricetidae* [43], which are found in the old and new world, respectively. *Muridae* is divided into multiple subfamilies, where the subfamily *Murinae* includes rats and mice [29,44] Rodents in this subfamily were shown to carry orthohantaviruses such as HTNV, SEOV, DOBV and THAIV(Thailand virus) [35,42,45]. The second family, *Cricetidae*, is also divided into several subfamilies, where the *Arvicolinae* subfamily includes voles and lemmings [29,44]. These small rodents are natural carriers of PUUV and TULV. [35,45]. A notable exception is the Thottapalayam virus (TPMV), as it is carried by insectivores of the *Cerocidurinae* subfamily, containing white-toothed shrew. These rodents are mainly found in India [46].

Orthohantavirus infection has historically been prevalent in Asia for the long time. China is the country where more than 90% of HFRS cases are found [45,47,48]. Approximately 300–500 HFRS cases are encountered each year in Korea [48]. The SEOV is believed to be found in multiple countries due to the widespread distribution of the domestic rats, a reservoir host. Still, SEOV infection is more common in Asia. Supporting this assumption is finding 14-34% of rats positive for anti-SEOV antibodies in Vietnam and Singapore[48,49]. Orthohantavirus infection cases were also reported in Middle East countries, where TULV-like orthohantavirus was isolated in the North West Iran outbreak [50]. In another study, a new orthohantavirus strain Serang virus (SERV) circulating in an Asian house rat in Indonesia was identified [51]. This virus was identified as related to TPMV circulating in Thailand. The majority of the PUUV infection diagnosed in Europe[9], with approximately 10,000 reported cases each year. Till now, approximately eight lineages of *Puumala orthohantavirus* are discovered across different countries of Europe and far east Russia (Table 1) and their geographical locations are represented with the help of map in figure 4.

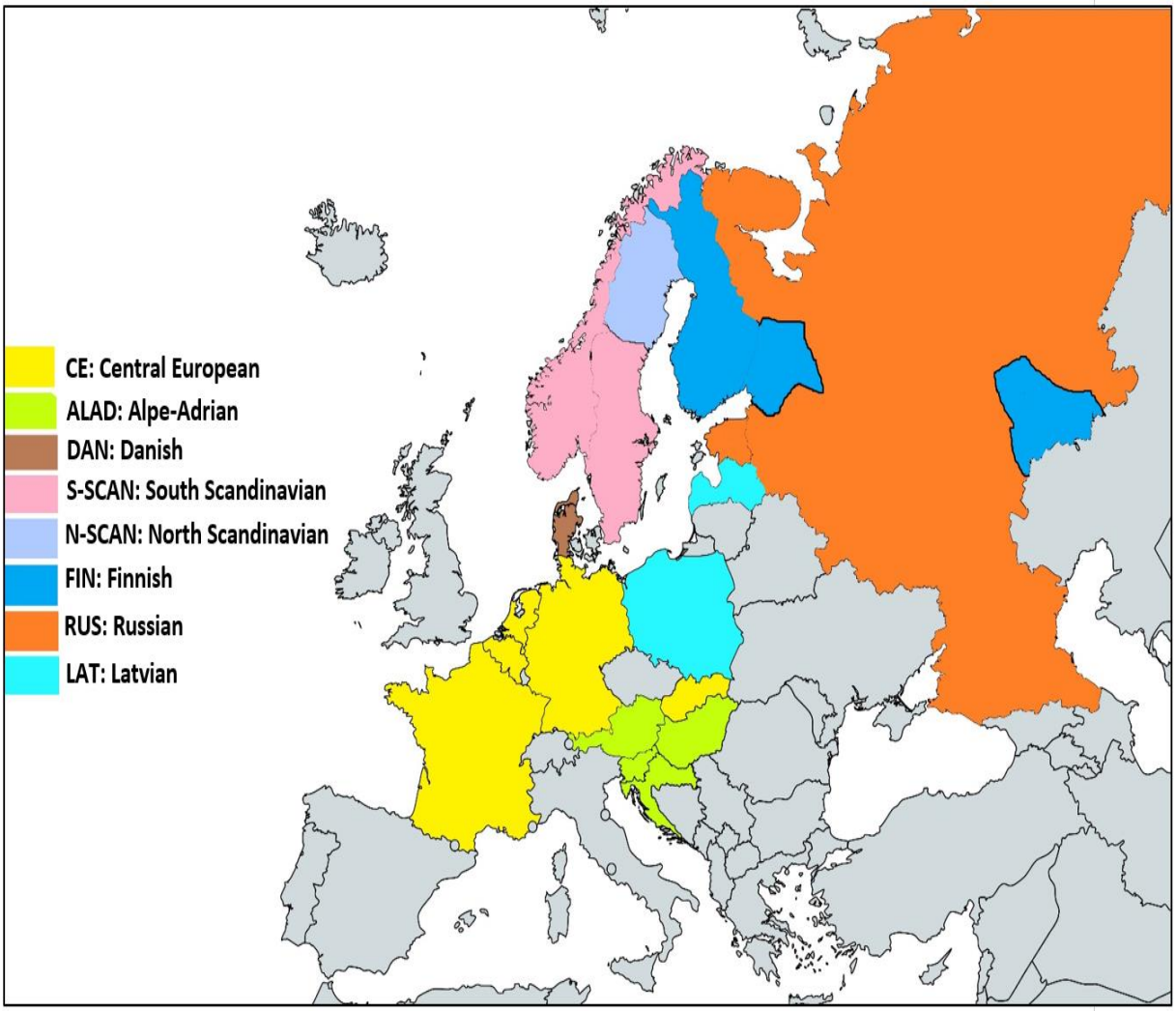

Figure 4: Geographical representation of eight lineages of PUUV. The group of lineages found in each country is marked with different colors**.**

2.6 Prevention of orthohantavirus infections

Treatment of orthohantavirus infection is supportive and not specific [52,53]. Therefore, the main form of orthohantavirus control is prevention of infection. Prevention measures include two methods: (1) minimizing exposure to rodents and (2) vaccination.

2.6.1 Minimizing rodents exposure

Exposure to virus contaminated aerosol or direct contact with rodents or their excreta are routs of orthohantavirus infection[54,55]. To minimize this exposure, measures should be made to prevent small rodent entry into the building [56–58]. Also, rodent control inside as well as around home and work area will reduce contact with infected small rodents. Rodent urine and droppings should be removed [59].The procedure starts with ventilation of the space for at least

30 minutes [60]. Then, wearing protective clothing, urine and dropping should be sprayed with disinfectant and soaked before the removal.

Monitoring of rodent population by the local authority is essential for prediction of the orthohantavirus outbreaks. There are multiple small rodents identified in Asia, which could carry HNTV, SEOV and THAIV [61]. These small rodents include *A. agrarius*, the main reservoir host for HTNV [62], and *Rattus norvegicus,* carrier for SEOV [63]. A novel THAIV Orthohantavirus was found circulating in *Bandicota indica, Rattus rattus* and *Eliurus majori* rat species [64–66].Studies have shown that the density of the population could affect horizontal transmission of the orthohantavirus increasing number of infected rodents [67,68]. A higher number of infected small rodents could potentially increase the chance of the human contact and exposure to orthohantavirus. It was also shown that there are threshold density of rodent population is required to maintain orthohantavirus [67,69,70].

2.6.2 Vaccination

There is no World Health Organization (WHO) or FDA approved vaccine for prevention of orthohantavirus infection. Likewise, orthohantavirus vaccine is not approved by the local authorities in endemic areas such as Europe, North and South Americas [71]. However, there are inactivated orthohantavirus vaccines licensed in China and Korea [72]. Inactivated rodent brain used in HFRS vaccines which is authorized to be used in China [73,74]. The first inactivated orthohantavirus vaccine was approved in 1993 in China [73]. Since then, four inactivated HTNV and SEOL based vaccines were used in China and demonstrated safety and efficacy of protection [75]. Starting 1990, this vaccine has also been used in Korea [76]. Since the beginning of the vaccination, numbers of HFRS cases reduced significantly [76]. Effectiveness of the vaccine was also reported by Park et al; however, authors state that using large cohort and long monitoring period is required to make conclusions regarding vaccine efficacy [77].

2.7 Types of orthohantavirus vaccines

2.7.1 Inactivated vaccines

These were the first type of vaccines containing inactivated orthohantavirus virion [78]. HNTV was propagated in the brains of suckling mice followed by chemical inactivation before tested for immunogenicity [79]. In 1990, this vaccine under the commercial name Hantavax was tested in clinical trial where safety and seroconversion of over 90% was demonstrated [80,81] Neutralizing antibodies were also demonstrated in 75% of vaccinated one month after the

booster [81]. Lesser antibody prevalence, 23 and 41%, as demonstrated in several studies [82,83]. Studies have demonstrated anti-orthohantavirus antibodies several months after immunization [82,84]. Also, the immune response was detected one year after immunization [80]. The protective efficacy of Hantavax vaccine was suggested to explain the decline in number of HFRS cases South Korea between 1991 and 1997 [78]. Later studies aimed to analyze the effect of Hantavax vaccine to effect the disease progression demonstrated the reduction of stage 3 acute kidney injury and requirement for the dialysis in vaccinated cohort [85]. In another study, the efficacy of anti-orthohantavirus vaccine was demonstrated in immunized cohort from Yugoslavia [86]. Also, the developers of Hantavax in collaboration with Yugoslavia research team demonstrated protective efficacy of the vaccine [77].

2.7.2 Virus-like particle (VLP) vaccine

Inactivated vaccine was shown effective to activate the humoral immune response. However, it appears that these type vaccines had substantial limitations such as failure to induce long-term antibody response; multiple immunizations and potential side effects. Therefore, there were still an interest in developing vaccines, which could address these obstacles. VLP vaccine could provide a solution to some of these limitations. Several VLP are nano-sized self-assembly competent structures made by viral proteins [87]. They have spike proteins, which could bind to the host receptor [88,89]. Therefore, VLP host cell entry resembles the natural infection. However, VLPs lack nucleic acid, rendering them incapable of replication [87]. As the result, VLPs could deliver the viral antigens to the host cells without virus replication and, subsequent, disease symptoms. This feature of VLP made them an attractive tool for development of the second-generation vaccines.

The efficacy of the VLP containing HNTV N and Gn/Gc proteins was tested in mouse model [90]. These chimeric VLPs induced humoral and cellular immune responses, which were stronger as compared to HNTV VLP or commercially available inactivated vaccine. It should be noted that chimeric VLPs also protected mice from HTNV challenge. Similar results were obtained by Ying et al, using GM-CSF-CD40L chimeric VLP [90]. Additionally, authors have demonstrated that incorporation of GM-CSF-CD40L stimulated macrophages and dendritic cells. In another study by Dong et al, chimeric VLPs were shown to induce long-term immune response with neutralizing antibodies circulating six months after immunization [91].

Production of anti-orthohantavirus antibodies after immunization with chimeric hepatitis B virus (HBV) particles containing PUUV N protein polypeptide [92]. These chimeric HBVs expressing PUUV N protein polypeptide induced protective immune response in bank voles,

natural reservoir of the orthohantavirus [93]. In another study, the immunogenic efficacy of HBV core particles carrying the N protein polypeptide of the DOBV, HNTV or PUUV in mice model was demonstrated [94] These chimeric particles induced high titer of cross reactive antibodies[95].

2.7.3 DNA vaccine

DNA vaccines have multiple advances as compared to inactivated vaccines. DNA vaccines are safe, as they are replication defective. They are also nonvirulent and fail to produce clinical symptoms of the disease. As early as 1992, the HNTV DNA vaccine expressing N and G proteins was developed using vaccinia virus as a vector [96]. This vaccine elicited protective immune response in hamster model. The same vaccine was later shown to be protective against HNTV, SEOL and PUUV [97]. Interestingly, anti-HNTV neutralizing antibodies were detected in immunized animals, while, in contrast, there were no antibodies to SEOL virus. In clinical trials phase I, neutralizing antibodies were demonstrated in immunized individuals. However, previous exposure to vaccinia virus appear to interfere with the efficacy of developing neutralizing antibody in volunteers [94].

DNA vaccines appear to be immunogenic and induce neutralizing antibody response. For example, two DNA vaccines, HTNV and PUUV, were tested in phase I clinical trial [98]. Neutralizing antibodies were elicited by both vaccines, however, only about half of vaccinated were seropositive. DNA vaccine against SEOL induced antibody response in Syrian hamsters and protected against infection [99]. Rhesus monkey immunization with another DNA vaccine, coding for HNTV and ANDV M genes, was shown to induce neutralizing antibodies [100]. Neutralizing antibodies can bind to envelop protein and prevent viral entry [101,102]. This reduces virus infectivity and prevent it dissemination. Therefore, ability to elicited neutralizing antibodies could be used to evaluate the efficacy of the vaccine [103,104].

2.7.4 Subunit vaccines

Orthohantavirus Gn/Gc and N proteins could elicit strong humoral immune response [105–107]. Gn/Gc proteins could induce neutralizing antibody response [108], while N protein activates non-neutralizing antibody [109]. Additionally, N protein stimulate T cell immune response [110,111], also shown to induce the cross reacting immune response [112,113]. Similarly, recombinant DOBV N protein was shown to induce antibodies cross-reacting with PUUV and HNTV in mice [114]. In another study, immunization with SEOL recombinant NP was shown to induce high-titer antibody[115]. Recently, the efficacy of eliciting humoral and T cell immune response by delivery of PUUV Gn/Gc and N protein using microvesicles was

demonstrated [116]. However, the efficacy of these recombinant proteins protection against lethal infection remains to be determined[117].

2.8 Diagnosis of orthohantavirus

The diagnosis in endemic areas is often based on clinical symptoms and presence of IgM. However, the expertise of the healthcare provider is essential for early diagnosis and appropriate treatment. As Kim and Han reported, 54% of HFRS patients were misdiagnosed at the admission [118] due to unusual symptoms. These led to longer hospitalization as compared to patients with HFRS diagnosed early after admission. HFRS could be misdiagnosed as an acute abdomen [119,120].It was suggested that this diagnosis could lead to unnecessary surgery and potential life-threatening complications. In another study, analysis of 1250 HFRS cases revealed that 13.2% were diagnosed with acute abdomen [121]. Authors suggest that fibrogastroduodenoscopy and diagnostic laparoscopy is optimal for differential diagnosis.

Another challenge could be HFRS diagnosis in children, Because disease is commonly mild in this age patients [122]. Zhang et al had demonstrated two cases of HFRS in children with atypical symptoms [123]. The initial differential diagnosis with systemic lupus erythematosus was done in these patients. Also, differential diagnosis with leptospirosis, rickettsiosis and heart failure is suggested [124,125] Leptospirosis is one of the most commonly considered for differential diagnosis with HFRS. This is explained by the pathology of leptospirosis where tubule-interstitial nephritis and thrombocytopenia are common [126]. Damage to kidney tissue and decreased thrombocyte counts are also commonly found in HFRS, making clinical symptoms of these diseases fairly similar. That could be the reason for misdiagnosis of HFRS, especially in low HFRS endemic regions. This assumption was tested by Groeijenbier et al where 4.3% and 4.1% cases diagnosed as leptospirosis were shown to have anti-orthohantavirus antibody [127].

2.9 Treatment of orthohantavirus

There is no specific post exposure therapeutics for HFRS. Therefore, treatment may differ between healthcare facilities adapting patient management protocols based on the local regulatory authority's recommendations. Therapeutics used for treatment of HFRS could be classified as targeting virus, immune response and supportive therapy.

Therapeutics targeting virus could: block virus entry and replication. Neutralizing antibodies can bind to surface glycoproteins, preventing binding to integrin receptors. Transfer of

orthohantavirus convalescent serum containing neutralizing antibodies was shown to have protective effect in non-randomized multicentre trial [128]. Also, the protection efficacy of convalescent serum was confirmed in *in vivo* experiments [129,130] Several monoclonal antibodies with neutralizing activity were generated against HNTV [130,131]. Phase I and II clinical studies has demonstrated the therapeutic efficacy of these neutralizing antibodies in the early stage of HFRS [130,132].

Novel therapeutic approaches targeting orthohantavirus entry are still in development stage. One of them is based on use of the peptides binding to αvβ3 integrin receptors [133]. Orthohantaviruses bind to this integrin receptor for the cell entry [134]. Therefore, initial interaction between orthohantavirus and integrin receptor is crucial moment in virus replication process. By preventing this interaction, virus entry could be abrogated protecting cells from infection. Supporting this hypothesis was study by Song et al where monoclonal antibodies to β3 integrin protected mice from HNTV infection [135]. This concept was further developed by Hall et al., where peptides binding to the integrin receptor were shown to block orthohantavirus entry [133]. The efficacy of neutralizing SNV, ANDV and HTNV by selected cyclic nonapeptides was demonstrated in follow up study [136]. *In vivo* studies could evaluate the therapeutic efficacy of these peptides against orthohantaviruses.

Another approach targets virus replication. One of the earliest drugs tested for its therapeutic efficacy against the orthohantavirus infection is Ribavirin. Ribavirin is nucleotide analog (1-beta-D-ribofuranosyl1,2,4-triazole-3-carboxamide) used for treatment of HFRS [137]. The main mechanism of anti-viral activity of this drug is induction of mutation into the viral RNA leading to fatal errors [138]. Clinical trials demonstrated the reduced morbidity and mortality in HFRS when treatment was initiated early after HNTV exposure [137]. However, later initiation of treatment was less effective. Lack of the Ribavirin therapeutic efficacy was reported when used for treatment of PUUV infected patients[139]. Favipiravir is antiviral drug selectively inhibiting the RdRp of the negative strand segmented viruses [140]. The efficacy of favipiravir was demonstrated against SNV and ANDV *in vitro* as well as *in vivo* [141]. Similar to ribavirin, the protective effect of this drug was demonstrated only when used at the early stage of infection. Late administration failed to reduce virus load and protect hamsters from lethal ANDV infection.

Corticosteroids are most commonly used for treatment of orthohantavirus infection for their anti-inflammatory effect [142]. Also, decreased level of corticosteroids was demonstrated in

orthohantavirus infected rats [143] suggesting the role of these hormones in pathogenesis of this infection. Data from HNTV and PUUV infected patients supported the hypothesis of corticosteroids contribution to pathogenesis of HFRS. Damage to hypothesis and adrenal gland could lower cortisol level and, potentially, contribute to inflammation [144,145]. This data supported the inclusion of corticosteroids in to the HFRS treatment protocol. Rapid recovery was demonstrated in two severe PUUV cases after administration of corticosteroids [146]. Also, thrombocyte counts were restored in NE patient after prednisolone treatment was initiated [147]. However, no therapeutic effect of corticosteroids in ANDV infected patients was reported in clinical trial [148].

Chapter 3: Objectives

- Identification of conserved peptide containing multiple epitopes.
- Interaction analysis of peptides with Human leukocyte antigens(HLA) alleles.

Chapter 4: Material and Methods

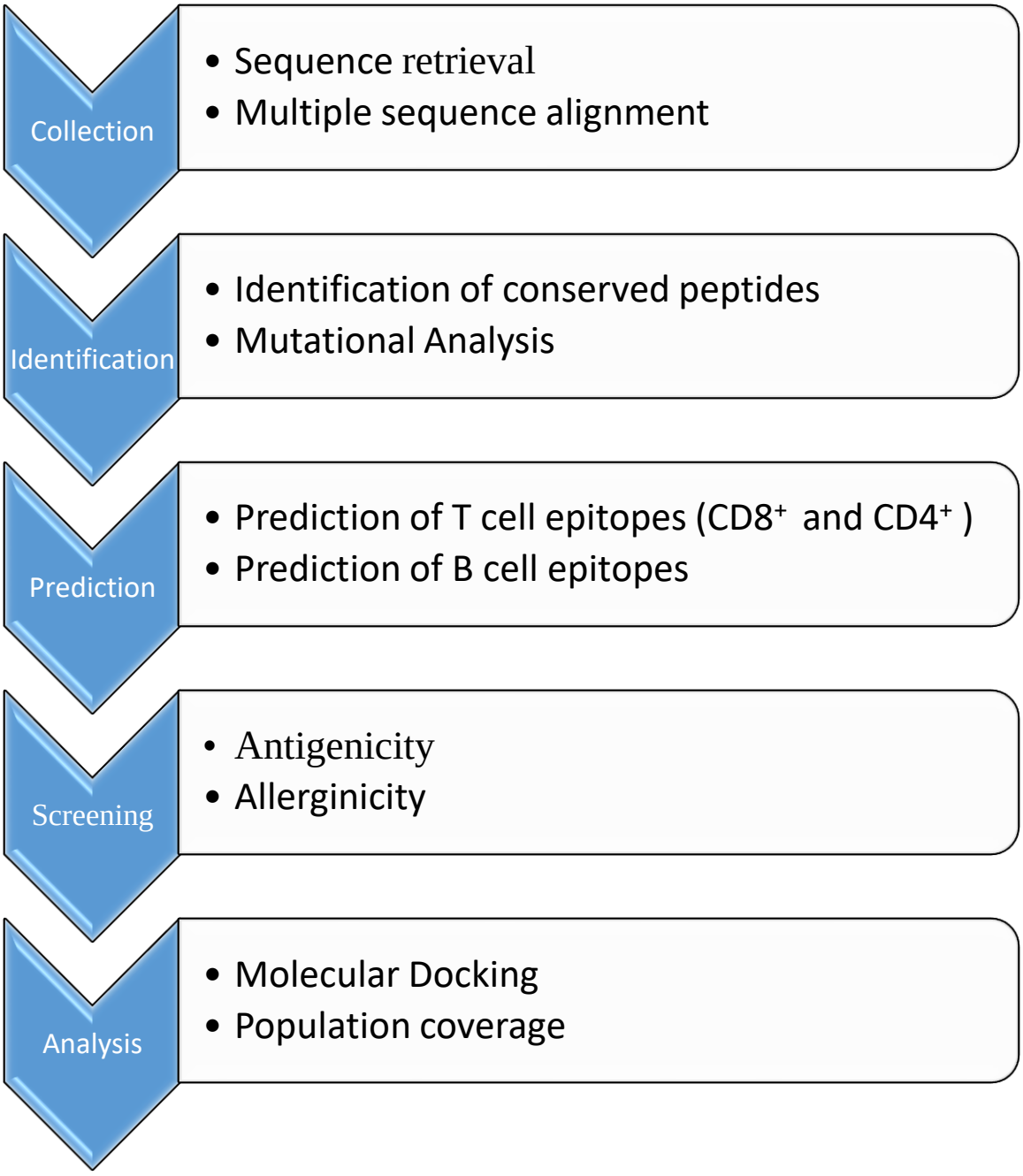

Figure 5: Flowchart depicting the methodology

4.1 Sequence retrieval and multiple sequence alignment

Complete nucleocapsid protein sequences (433 amino acids) belonging to *puumala orthohantavirus*, of the human host were retrieved from the NCBI database in FASTA format. Further, the redundant sequences were removed using Jalview software [149] at a 100 threshold value. The retrieved nucleocapsid protein sequences were aligned using Multiple Sequence Comparison by Log-Expectation (MUSCLE) tool in MEGA X (Molecular Evolutionary Genetics Analysis) software [150]. Output file was downloaded in FASTA format.

4.2 Mutational and conservation analysis

The Antigen Variability analyzer (AVANA) tool [151] was used to determine the conserved regions of the protein, based on ethereum algorithm. Conserved peptide fragments were identified at 90% conservancy using MSA file as an input. Conserved peptides showing homology in seven to nine consecutive amino acids with human proteome were considered as autoimmune peptides. These sequence were analyzed by using protein Basic local alignment search tool (BLASTp) online server [152]. Multiple sequence alignment (MSA) file was used as an input source. Mutational analysis of the nucleocapsid protein sequence of *Puumala orthohantavirus* with respect to its first human host sequence was performed using the BioEdit software version 7.2.5 [153]. As NCBI reference sequences with human host has not been reported but NCBI reference sequence with rodent host is available. Therefore pairwise alignment using EMBOSS[154] was carried out to evaluate similarity and identity of first human host sequence with available NCBI reference sequence. Then mutational frequency at each position was calculated using formula, No of mutation at a position/Total number of sequences. Further, mutations recorded in Bioedit software were located in 90% conserved fragment and analyzed for conservancy percentage in the sequence dataset using AVANA. Therefore, 90% conserved peptides devoid of mutations and autoimmune regions were considered for prediction of multiple epitopes.

4.3 Prediction of T and B cell epitopes

T cell epitope were predicted by using tools NetMHCpan-4.1server [155] and NetMHCIIpan-4.0[156] for $CD8^+$ T cell (HLA class I) and $CD4^+$ T cell(HLA class II), respectively . In order to predict $CD8^+$ T cell epitopes, the threshold value for the strong binders was 0.5, while the threshold value for weak binders was 2. Similarly, for $CD4^+$ T cell epitopes, threshold value for strong binders was 1(% rank) and the threshold value for weak binders was 5(% rank). Overlapped peptide fragments were generated which contained multiple $CD8^+$ and $CD4^+$ T cell

epitopes. Furthermore, B cell epitopes were identified with the help of IEDB (Bepipred linear epitope prediction 2.0)[157], using class II overlapped fragments as an input.

4.4 Screening of peptide for antigenicity and allergenicity

A screening of the final peptides against antigenicity and allergenicity was performed. The two online servers: Vaxijen v2.0 server[158] and Algpred [159], were used to check their responses. Vaxijen v2.0 server used for the assessment of antigenicity, which is based on auto cross-covariance (ACC) for virus antigens and this is carried out at 0.4 threshold. AlgPred used for the assessment of Allergy, based on Motif Alignment & Search Tool and IgE epitopes in query protein sequence.

4.5 Molecular Docking

The interaction between human leukocyte antigen (HLA) and the identified peptides was determined using molecular docking. HPEP dock server [160] was used for molecular docking. Crystal structure of 10 supertypes of HLA Class-I and 10 supertypes of HLA Class-II bound to their native peptide were downloaded from the Protein data bank(PDB) in PDB format, selected based on their high-resolution values. Using Discovery studio software, native peptides were separated from the HLA crystal structure. Following this, refinement and energy minimization was carried out for the native peptide and HLA structures using tools Galaxy server [161], 3D refine [162] and Chimera[163]. HLA and bound peptide in HLA crystal structure were separated and re-docked and considered as native peptide molecule (NP), and their docking score was used as a reference.

3D structure of identified peptide epitopes of both HLA Class-I and Class-II were generated by using the Modpep tool[164], and these peptides were used as test peptides. Following this, refinement and energy minimization was carried out for the test peptide using 3D refine and Chimera tools, respectively. Molecular docking was performed to determine the interaction between HLA and the test peptide by using Hpep dock. The pymol tool [165]was used to visualize the peptide-HLA interactions in the docked complex.

4.6 Population coverage Analysis

Population coverage analysis was done by using the IEDB population tool [166]. The tool estimates coverage of the identified peptide fragments in 16 geographical regions. List of HLA alleles displaying affinity for identified peptides were recorded and based on HLA alleles data population coverage was evaluated.

Chapter 5: Results

5.1 Sequence retrieval and multiple sequence alignment

81 complete nucleocapsid (N) protein sequences belonging to *Puumala orthohantavirus* of human host were downloaded from NCBI database in FASTA format reported till 5th August, 2021. 45 unique sequences were obtained after removing the redundant sequences which were. considered for further analysis.

5.2 Mutational and conservation analysis

For the Identification of conserved peptide, AVANA was performed using an MSA file as input and conservancy were checked at 90%. Initially, five peptides with 90% conservancy were obtained which were checked for probable identity with human protein. Four conserved peptides have shown partial identity with human immunoglobulin heavy chain, Zinc finger proteins and Myosin(MYH6) proteins. which were removed, and seven conserved peptides were obtained (Table 1). Mutational analyses were carried out by comparing the PUUV N protein sequences with respect to first human host sequence [QEH04716.1], collected in 1986, as a NCBI reference sequence with human host is not reported. Therefore, similarity and identity between rodent NCBI reference sequences and first human host nucleocapsid protein was evaluated using EMBOSS. First human host sequence displayed 99% similarity and 96.5% identity with rodent NCBI reference sequence [NP_941984.1]. Highest mutational frequency was observed in the range of 0.81-1.0, which shows the change in 15 amino acid positions and also found in two domains (cytoplasmic and non-cytoplasmic domains) and transmembrane regions as shown in figure 5 (Table 2). These mutations were also located in the conserved fragments, so the conserved fragments were further analysed by comparing the results of bio edit and AVANA software. It was found that mutated amino acids allocated in the conserved fragment exhibited >90% conservancy among 45 sequences (Table 4). Therefore, those mutated amino acids can be considered conserved in the population. However, with respect to the reference sequence, that amino acid position is mutated, but with time, it has been mutated and become conserved.

Table 1: Conserved peptide of *Pummula orthohantavirus* nucleocapsid protein

	Conserved peptides without Autoimmunity	Location	Length
1	VARQKLKDAEKAVEMDPDDVNKNTLQARQQTVSALEDKLADFKR	20 to 63	44
2	MADAVSRKKMDTKPTDPTGIEPD	65-87	23
3	SLRYGNVLDVNAIDIEEPSGQTADWYTIGVYVIGFTLPIILKALYMLSTRGRQ TVKENKGTRIRFKDDTSFEDINGIRRPKHLYVSMPTAQSTMKAEELTPGRFR TIVCGLFPTQIQVRNIMSPVMGVIGFSFFVKDW	95-232	138
4	LDKNHVADIDKLIDYAAS	277-294	18
5	PNAPWVFACAPDRCPPTCIY	306-325	20
6	AGMAELGAFFSILQDMRNTIMASKTVGTA	327-355	29
7	SSFYQSYLRRTQSMGIQLDQRIILLYMLEWGKEMVDHFHLGDDMDPELRGL AQALIDQKVKEISNQEPLKI	363-433	71

Table 2: List of Highest mutational frequency

Mutations	Position	No of mutations	Mutational Frequency
E>D	10	44	0.97
R>K	30	43	0.95
V>M	34	42	0.93
Y>D	35	44	0.97
Y>F	61	42	0.93
I>L	131	44	0.97
P>A/T/S/I	233	43	0.95
E>D/N	234	43	0.95
K>R	235	44	0.97
V>T/A/I	260	39	0.86
R>K	267	43	0.95
V>I	268	38	0.84
T>Q	272	44	0.97
D>N	302	43	0.95
S>T>A	416	44	0.97

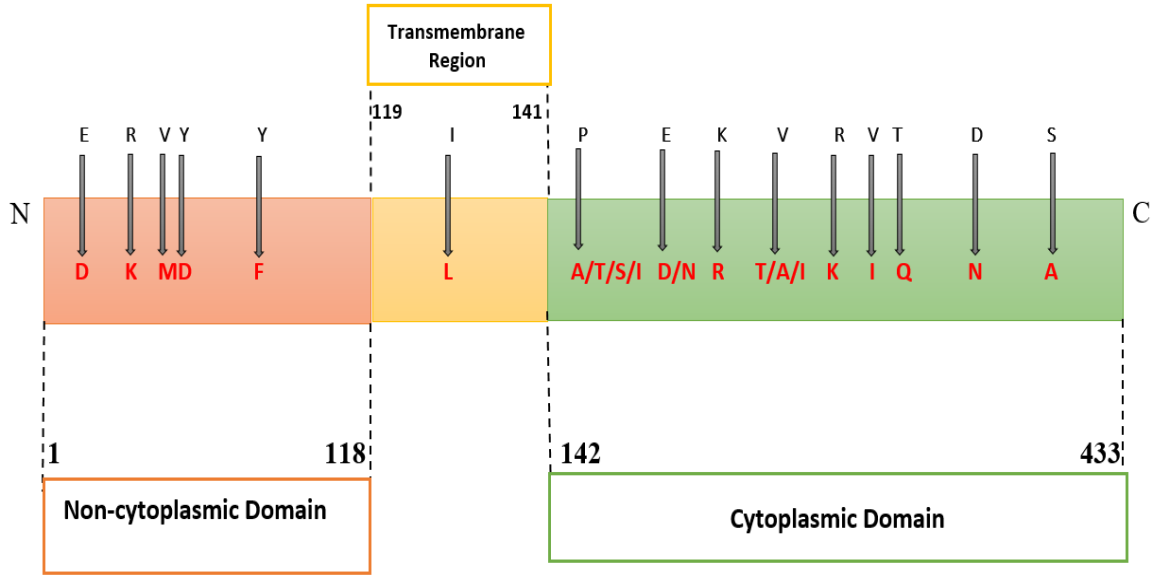

Figure 5: Schematic representation of highest mutational frequency falling in two domains and transmembrane regions. The identified mutations are highlighted in red color.

Table 3: Represents the conservancy of conserved peptides

S.no	Conserved Peptide (Location)	Mutation	% Conservancy of residue (AVANA)*	%Conservancy of residue (Bioedit7.2)*
1	1 (20 to 63)	R30K	95.56%	4.44%
		V34M	93.33%	6.67%
		Y35D	97.78%	2.22%
		Y61F	93.33%	6.67%
2	2 (95-232)	I131L	97.78%	2.22%
3	3 (363-433)	S416A	93.33%	4.44%
		T416A	97.78%	2.22%

***Name of tool in which those mutations were recorded.**

5.3 Prediction of T and B cell epitopes

T cell epitopes were predicted by using tool NetMHCpan 4.1 server and NetMHCIIpan 4.0 for $CD8^+$ T cell and $CD4^+$ T cell, respectively. HLA Class-I complexes are present on surface of the nucleated cells and recognizes $CD8^+$ T cells, The binding groove of class I molecules is closed because of the presence of conserved tyrosine residues at both ends; this reduces the size of the binding groove, and only eight to ten residues can be bound in this groove [167]. Hence nonamer peptide length were considered for prediction of $CD8^+$ epitopes. On the other hand, HLA Class-II is recognized by antigen presenting cells (dendritic cells and macrophages) and activates $CD4^+$ T cells. It has an open binding groove, So it can bound peptide up to 13-25 residues length in its binding pocket during docking [167], Seven obtained conserved peptide fragments were used as an input for the prediction of $CD8^+$ T cell epitopes with 2915 HLA class I alleles. 102 $CD8^+$ T cell epitopes were predicted (data in appendix-A) and five peptides containing overlapping $CD8^+$ T cell epitopes were selected (Table-5). These five overlapped peptides were used as an input for prediction of $CD4^+$ T cell epitope prediction using NetMHCIIpan 4.0 where 681 HLA class II alleles were taken into account.

55 $CD4^+$ T cell epitopes (data in appendix-B) were obtained as strong binders and epitopes predicted for ≥ 20 HLA alleles were selected to form overlapping peptides, As a result five peptide fragments having overlapping $CD4^+$ T cell epitopes were selected (Table-6). Further, analyzed the presence of B cell epitopes in these five peptides using the IEDB tool. Three peptides ((P1, P2 and P4) were showing the presence of B cell epitopes. These three peptides are the final peptide selected because it contains multiple epitopes of both T and B cells (Table-7)

Table 4: Peptide fragments containing multiple epitopes of $CD8^+$ T cell

Peptide fragments of HLA class I	location	Length	No. of epitopes
VARQKLKDAEKAVEMDPDDVNKNTLQARQQTVSALEDK	20-57	38	12
LRYGNVLDVNAIDIEEPSGQTADWYTIGVYVIGFTLPIILKALYMLSTRG RQTVKENKGTRIRFKDDTSFEDINGIRRPKHLYVSMPTAQSTM	96-188	93	31
AEELTPGRFRTIVCGLFPTQIQVRNIMSPVMGVIGFSFFVK	190-230	41	17
GMAELGAFFSILQDMRNTIMASKTVGTA	328-355	28	10
SSFYQSYLRRTQSMGIQLDQRIILLYMLEWGKEMVDHFHLGDDMDPEL RGLAQALIDQKVKEISNQEPLKI	363-433	71	23

Table 5: Peptide fragments containing multiple epitopes of CD4+ T cell

Peptide Fragments of HLA class II	Location	Length	No of epitopes
RQKLKDAEKAVEMDP(P1)	22-36	15	1
ILKALYMLSTRGRQTVKENKGTRIRFKDDTSFEDING(P2)	134-170	37	8
RRPKHLYVSMPTAQSTM(P3)	172-188	17	3
TQSMGIQLDQRIILLYM(P4)	373-389	17	3
MDPELRGLAQALIDQKV(P5)	406-422	17	3

Table 6: Final peptide fragments containing multiple epitopes of both T and B cells

S. No	Peptides	$CD8^+$ Epitopes	$CD4^+$ Epitopes	B –cell Epitopes
1	RQKLKDAEKAVEMDP(P1)	RQKLKDAEK(A1) KLKDAEKAV(A2) KDAEKAVEM(A3) AEKAVEMDP(A4)	RQKLKDAEKAVEMDP(P1)	KDAEKAV
2	ILKALYMLSTRGRQTVKENK GTRIRFKDDTSFEDING(P2)	KALYMLSTR(B1) LYMLSTRGR(B2) LSTRGRQTV(B3) STRGRQTVK(B4) GRQTVKENK(B5) TVKENKGTR(B6) IRFKDDTSF(B7)	ILKALYMLSTRGRQTVKE(P2) RGRQTVKENKGTRIRFKD(P2.1) GTRIRFKDDTSFEDING(P2.2)	STRGRQTVKENKGTRIRFKDDTSFE
3	TQSMGIQLDQRIILLYM(P4)	MGIQLDQRI(C1) IQLDQRIIL(C2) QLDQRIILL(C3) LDQRIILLY(C4) DQRIILLYM(C5)	TQSMGIQLDQRIILLYM(P3)	IQLDQRII

5.4 Peptide having antigenic properties but devoid of allergenicity

Screening of the final peptides for antigenicity and allergenicity were performed. The two online servers: Vaxijen v2.0 server and Algpred were used to check their responses. Three peptides (P1, P2 and P4) are found to be antigenic, when analyze under the tool Vaxijen v2.0 and these peptides also displayed the non –allergic response when analyze under the tool Algpred.

Table 7: Screening of identified peptide fragments against Antigenicity and Allergenicity

Peptide	Antigenicity	Allergenicity
RQKLKDAEKAVEMDP(P1)	1.1827 (Probable ANTIGEN)	Non- Allergen
ILKALYMLSTRGRQTVKENKGTRIRFKDDTSFEDING(P2)	0.6283 (Probable ANTIGEN)	Non-Allergen
TQSMGIQLDQRIILLYM(P4)	0.6368 (Probable ANTIGEN)	Non-Allergen

5.5 Molecular Docking

The binding interaction between the identified peptides and HLA class I and HLA class II molecules were analyzed using molecular docking. Due to the closed groove of the HLA class I, the nonamer epitopes falling in the identified peptides were docked with ten crystal structures of HLA molecules. However, class II molecules have an open binding groove, therefore P1, P4 were docked as such whereas the length of P2 is relatively large i.e. 37 , as per the literature the ideal length of peptides is 20-30 amino acids [168] but the length of peptide P2 have more than 30 amino acids, So, it was spilt into three peptide fragments (P2, P2.1, P2.2) containing multiple $CD4^+$ T cells epitope having only one overlapping amino acid Further, using the modpep server, the three-dimensional structure of epitopes and identified peptide were generated. Refinement and energy minimization of peptides structures was done before docking.

In Hpepdock, the docking energy score indicates the binding affinity of the protein-peptide complex. The docking score of the native peptide molecule (NP) was used as a reference. Selected epitopes/peptides-HLA docked complexes showing higher energy scores than reference docked molecules were considered good binding models. Ten HLA class I molecules were docked with native and test peptides and the majority of epitopes have displayed good binding interaction. (Figure 7A). Epitope A1(-165.908) have shown good binding interaction with HLA-B-37(NP-165.532). Three epitopes, A1, A2 and A4, have displayed the best docking score of -135.579, -114.675, and -120.734, respectively with the HLA-C-05 allele (NP-107.138), whereas A3 is the only epitope which displayed the less binding affinity for all HLA class I alleles. B1, B2, B3, C4 and C5 displayed higher average docking scores of - 176.03, -181.33, -161.183, -171.35, and -174.58, respectively than the average docking score of NP (-160.578) with ten HLA class I molecules. However, individually HLA-A-02 (NP -176.929) showed the highest docking score with epitopes B2 (-204.616) and C4(-207.726) (Figure 8 A, B). Similarly, ten molecules of HLA class II were docked with native and test peptides; most epitopes exhibit good binding interactions (Figure7B). The average docking score of P2, P2.1 and P4 are -175.2, -179.465 and -187.161, respectively, and have shown higher docking scores than the native peptide docked molecule (NP -166.059) with ten molecules of HLA class II alleles. In contrast, P1(-123.488) have shown the least binding interaction with all HLA alleles. Individually HLA-DQ2 (NP -154.545) has shown the best binding affinity with P2.1(-244.994) and P4 (-281.681). (Figure 8 B, C).

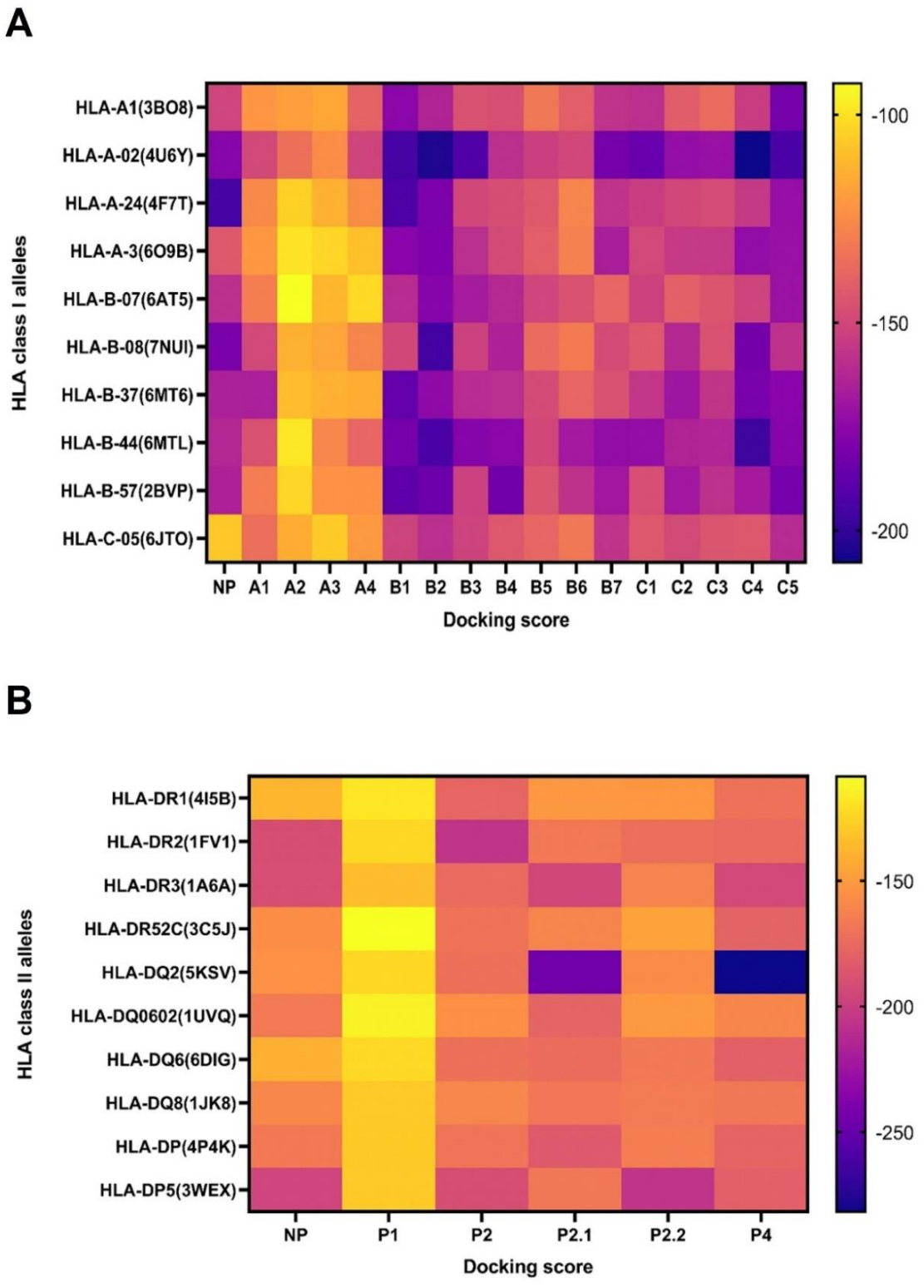

Figure 7: Heat map represents docking scores **(A)** Native peptide(NP) and test epitopes with HLA class I alleles **(B)** Native peptide(NP) and test peptides and epitopes with HLA class II alleles.

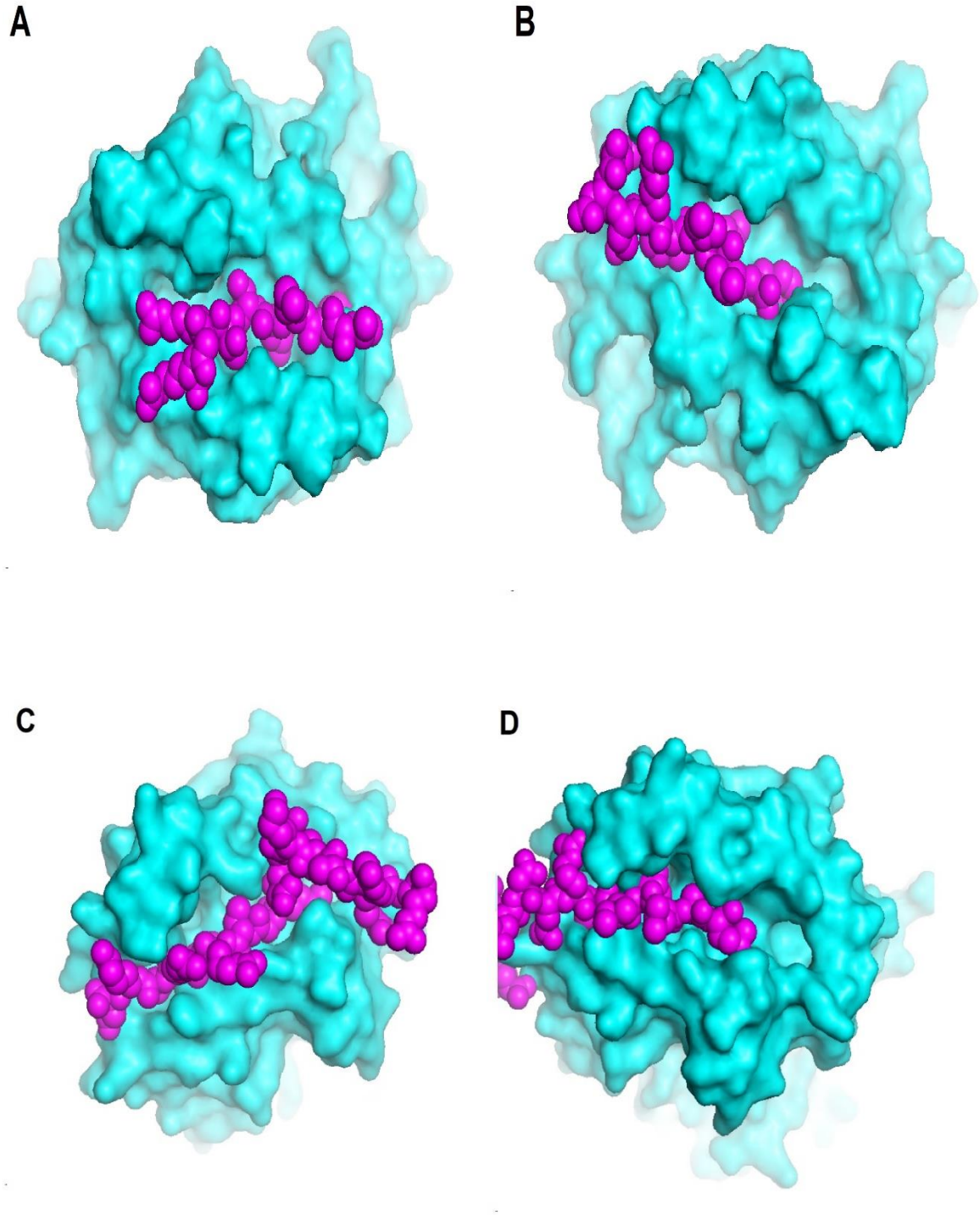

Figure 8: Poses of docked HLA-peptide complex displaying highest docking score. HLA is shown in cyan colour and epitopes and peptides in magenta colour **(A)** B2 epitope docked with HLA-A-02 (Docking score: -204.616), **(B)** C4 epitope docked with HLA-A-02 (Docking score: -207.726), **(C)** P1 docked with HLA-DQ2 (Docking score: -244.994) and **(D)** P3 docked with HLA-DQ2 (Docking score: -281.681).

5.6 Population coverage analysis

Population coverage analysis is one of the essential steps during the designing of peptide vaccines , as the frequency of different types of HLA may vary in different geographic locations because of the polymorphic nature of HLA [169]. Hence, if one epitope-HLA complex shows adequate population coverage in a particular region, that does not mean it will cover the same adequate population coverage in another region. In this study, the maximum population coverage of the three potential peptides (P1, P2 and P4) across six continents (Asia, Africa, Europe, North America, South America, and Australia) were analyzed. Maximum population coverage was found in Europe for all three peptides (99.67%, 99.99%, and 100%, respectively). In contrast to this the least population coverage was seen in North America for all three peptides (74.26%, 99%, 75.29%). In the P1 peptide, four regions showed population coverage higher than 90%, while some have coverage below 80%. In P2 peptide, all regions have more than 90% (>90%) population coverage. The majority of regions in P3 have shown 100% population coverage in Europe, South America, Australia. (Figure 8)

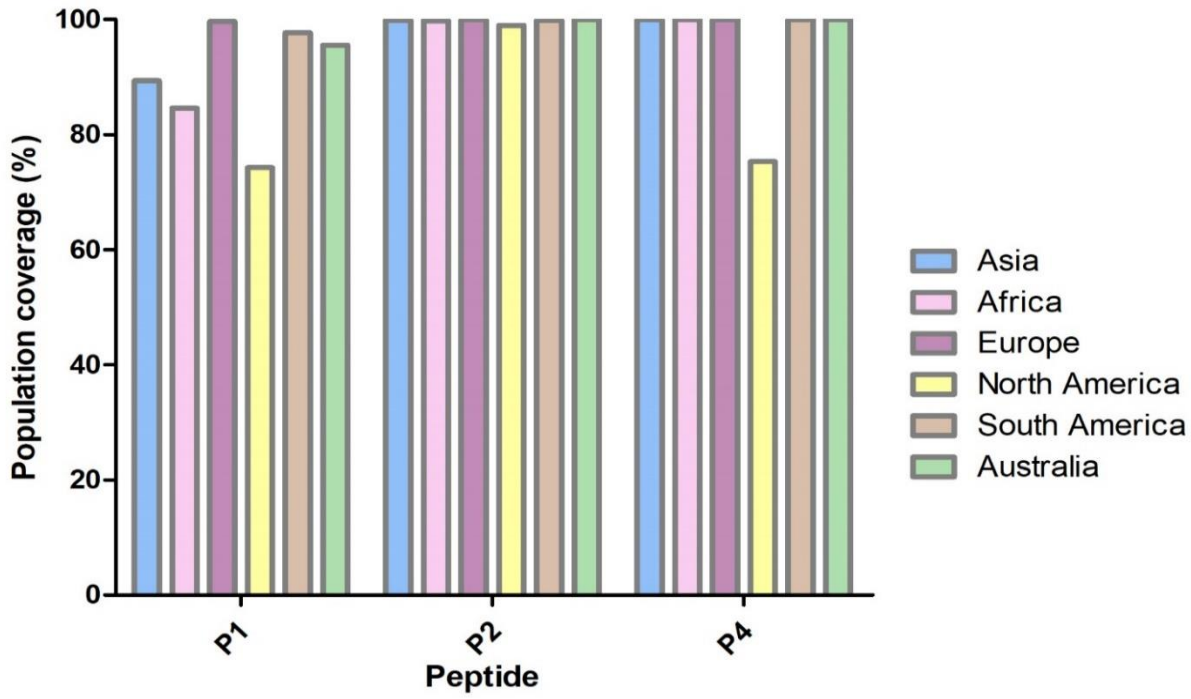

Figure 9: Graphical representing the Population coverage analysis of identified peptides (P1, P2 and P4) in six continents (Asia, Africa, Europe, North America (N.A), South America (S.A) and Australia) and average population coverage of identified peptides in six continental regions were >90%, followed the order P2 (99.88%) > P4 (99.49%) > P1 (90.50%).

Chapter 6: Discussion

Orthohantavirus is one of the emerging zoonotic virus, more than fifty Orthohantavirus species are presently known, out of which around twenty-four are pathogenic and causes two acute febrile diseases; HFRS and HCPS. Approximately 1,50,000 to 2,00,000 cases of Orthohantavirus infection reported annually across the world. Therefore, Orthohantavirus infection remains a serious threat to public health [47,170–172]. Currently, FDA has not approved any specific treatment or preventive measures to be used worldwide. Only inactivated vaccine is authorized to use against two strains of Orthohantavirus, i.e. Hantaan (HTNV) and Seoul(SEOV). In 1990, this vaccine under the commercial name Hantavax was tested in clinical trial where safety and seroconversion are over 90% was demonstrated [80,81]. However other nations stopped and resisted the development of this vaccine because the vaccine is made by growing formalin-inactivated HTNV on rodent brain cells. Also, this vaccine has some drawbacks; it elicits a low neutralizing antibody response, and after 2 to 6 months, booster doses are often required. In 1992, Using the vector (vaccinia virus) developed the HNTV DNA vaccine, expressing the nucleocapsid and glycoprotein proteins. Later this vaccine was found to be effective against other strains of Orthohantavirus (PUUV and SEOL), Though a study conducted by McClain et al. reported that vaccinia virus obstructs the efficacy of neutralizing antibody response [94].Another popular vaccine is peptide based vaccine, which is safe and more specific since it uses a specific peptide fragment of a disease-causing agent to stimulate the immune response. The majority of peptide-based vaccines are in Phase I and Phase II of clinical trials against Malaria, HIV, anti-plasmodium, Alzheimer's disease, anticancer and influenza virus[173]. Many anticancer vaccines based on synthetic peptides have made up to different phases of clinical trials. PR1 leukemia peptide vaccine against leukemia is in phase III trial. Degarelix (LHRH antagonist) against Prostatic neoplasms have made it to Phase IV[174]. Several research has recently been undertaken to develop multiepitope vaccine candidates against various strains of Orthohantavirus. Immunoinformatic approaches were conducted to design an effective multiepitope vaccine against Hantavirus and concluded that multiepitopes of glycoprotein and nucleocapsid protein could serve as a potential peptides in designing the vaccine candidates [175,176]. This study identified the conserved peptide fragments of nucleocapsid protein of *Puumala orthohantavirus*, and identified peptides were screened for autoimmunity; Elimination of the autoimmune sequence is essential if identified peptide shows any homology with the human proteome may trigger autoimmunity and cross-reactivity responses and further may also lead to vaccine failure.

Vaccination can trigger the autoimmune response; supporting this statement, Sasaki et al., reported that the influenza virus vaccine could induce clinical symptoms of autoimmune diseases such as Autoimmune hepatitis(AIH) [177]. In addition, some researchers have also reported autoimmune diseases like Multiple Sclerosis (MS) and Guillain–Barre syndrome (GBS) [178,179]. Currently, developed Moderna-vaccine against Covid-19 has also been reported to induce the autoimmune disorders. It is believed that the vaccine affects self-tolerance and causes autoimmune reactions through cross-reactivity[180]. In the current study, four conserved peptides have shown partial molecular mimicry with human immunoglobulin heavy chain, Zinc finger proteins and Myosin (MYH6) proteins. when employed to BLASTp [151]. Thus, these homologous sequences were removed in order to minimize the chance of an autoimmune reaction. These fragments were further employed for the multi-epitope prediction of T ($CD8^+$ and $CD4^+$) and B cells.

Epitope prediction using a computational approach is more efficient than the experimental method. The experimental epitope prediction method is time-consuming, expensive, not specific and requires antibody production to recognize the immunogenic region in the target protein. The use of computational-based epitope prediction can avoid the problems mentioned above[181]. HLA-peptide complex recognition on the cell surface by T cells elicits an immune response. Several computational tools are available for prediction of epitopes, and these tools are also efficient in analyzing the binding affinity of potential peptides with various HLA alleles. Several investigations have been conducted in these years using the NetMHCpan 4.1 server [182] and NetMHCIIpan 4.0 server[183]to predict $CD8^+$and $CD4^+$epitopes, respectively. These servers were employed to identify epitopes of HLA class I and HLA class II. Furthermore, the current study showed the strong binding interaction of identified peptides with HLA Class I and HLA Class II alleles. Most studies were carried out for epitope prediction of B cells using the IEDB linear B cell epitope prediction tool[184]. The identified peptides of T cells weree subjected to BepiPred linear epitope prediction. Finally, the three potential peptides (P1, P2 and P4) were generated by merging the overlapped peptide fragments. These peptides contains multiepitopes of T and B cells.

One of the advantages of peptide-based vaccine is that it minimizes the risk of side effects like allergic response. Vaccines are highly potent in causing allergic responses. Recently, developed vaccine against Covid -19 Pfizer-BioNTech has reported the cases of anaphylaxis [185]. Depending on the vaccination formulation, allergic reactions might range from mild to severe.In some studies, the protein was found to be a main source of allergen in the

vaccine[186]. So it is essential to remove those peptides which show allergenicity. In the current study, identified peptides were screened against allergenicity. Tool Algpred 2.0 [159]were used for the assessment, and all three identified peptides were found to be non-allergen and safe for further analysis.

The protein-peptide docking method is commonly employed to evaluate the vaccine's stability by examining its binding energies. In order to examine the molecular interaction between HLA and the identified Peptide, molecular docking and population analysis were performed. HPEP dock server[187,188] has been reported in many studies for docking of protein-peptide or HLA-peptide, their results evaluated in the form of docking score, representing the binding energy score of the Peptide with HLA molecules. This study employed an HPEP dock server to assess HLA-peptide interaction. Native peptide docking results were employed as a reference, and Identified peptide epitopes were used as test peptides. Generally, the test peptide docked model showing a higher docking score indicates the best interaction models. Most peptide epitopes have shown higher docking scores with diverse supertypes of HLA class I and HLA Class II when compared with native bound peptides, which are ideally showing strong binding affinity with HLA molecules.

The most popular tool for population coverage analysis is the IEDB population coverage tool[189]. This study used this tool to check the population coverage of three identified peptide fragments in six continents (Asia, Africa, Europe, North America, South America, and Australia). All three peptides have shown more than 90 per cent (>90%) population coverage. This study proposed the potential peptide molecules which can provide immunity against *Puumala orthohantavirus* strains. Therefore, it can be considered for the designing of peptide-based vaccine candidates against PUUV

Recently, several studies have been conducted to develop multiepitope vaccine candidates against various strains of orthohantavirus. In-silico approaches were conducted to design an effective multi-epitope vaccine against Hantavirus and concluded that multiepitopes of glycoprotein and nucleocapsid protein could serve as a potential peptide in designing the vaccine candidates.

Conclusion

Computational analysis results in three immunogenic peptide fragments P1, P2 and P4 of nucleocapsid protein. These peptides are highly conserved and contain multiple epitopes of both T ($CD4^+$ and $CD8^+$) and B cells. Further molecular docking and population coverage analyses proposed the potential peptides interacting with various HLA molecules. Therefore, these peptides can further be validated by in vivo experimental system to consider peptides as a vaccine candidate against Puumala orthohantavirus (PUUV).

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Appendix-A

Table A. $CD8^+$ T cell epitopes of *Puumala orthohantavirus* nucleocapsid protein

$CD8^+$ T cell epitopes					
VARQKLKDA	IEEPSGQTA	LSTRGRQTV	FRTIVCGLF	DRCPPTCIY	QLDQRIILL
RQKLKDAEK	VLDVNAIDI	STRGRQTVK	GLFPTQIQV	GMAELGAFF	LDQRIILLY
KLKDAEKAV	EPSGQTADW	GRQTVKENK	LFPTQIQVR	AELGAFFSI	DQRIILLYM
KDAEKAVEM	GQTADWYTI	TVKENKGTR	TQIQVRNIM	AFFSILQDM	QRIILLYML
AEKAVEMDP	TADWYTIGV	IRFKDDTSF	IQVRNIMSP	FFSILQDMR	IILLYMLEW
EMDPDDVNK	ADWYTIGVY	TSFEDINGI	QVRNIMSPV	ILQDMRNTI	YMLEWGKEM
DVNKNTLQA	DWYTIGVYV	DINGIRRPK	VRNIMSPVM	LQDMRNTIM	KEMVDHFHL
VNKNTLQAR	GVYVIGFTL	NGIRRPKHL	NIMSPVMGV	QDMRNTIMA	DHFHLGDDM
TLQARQQTV	YVIGFTLPI	GIRRPKHLY	IMSPVMGVI	MRNTIMASK	LGDDMDPEL
QARQQTVSA	IGFTLPIIL	IRRPKHLYV	SPVMGVIGF	NTIMASKTV	DMDPELRGL
ARQQTVSAL	GFTLPIILK	RPKHLYVSM	VMGVIGFSF	MASKTVGTA	ELRGLAQAL
QTVSALEDK	FTLPIILKA	HLYVSMPTA	GVIGFSFFV	SSFYQSYLR	AQALIDQKV
MADAVSRKK	TLPIILKAL	SMPTAQSTM	VIGFSFFVK	SFYQSYLRR	LIDQKVKEI
ADAVSRKKM	LPIILKALY	AEELTPGRF	NHVADIDKL	SYLRRTQSM	KVKEISNQE
VSRKKMDTK	IILKALYML	LTPGRFRTI	HVADIDKLI	RTQSMGIQL	KEISNQEPL
SRKKMDTKP	KALYMLSTR	TPGRFRTIV	ADIDKLIDY	MGIQLDQRI	EISNQEPLK
LRYGNVLDV	LYMLSTRGR	RFRTIVCGL	APDRCPPTC	IQLDQRIIL	ISNQEPLKI

Appendix-B

Table B. $CD4^+$ T cell epitopes in *Puumala orthohantavirus* nucleocapsid protein

$CD4^+$ T cell epitopes				
VARQKLKDAEKAVEM	ILKALYMLSTRGRQT	IRFKDDTSFEDINGI	PKHLYVSMPTAQSTM	RTQSMGIQLDQRIIL
ARQKLKDAEKAVEMD	LKALYMLSTRGRQTV	RFKDDTSFEDINGIR	AEELTPGRFRTIVCG	TQSMGIQLDQRIILL
RQKLKDAEKAVEMDP	KALYMLSTRGRQTVK	FKDDTSFEDINGIRR	AFFSILQDMRNTIMA	QSMGIQLDQRIILLY
EKAVEMDPDDVNKNT	ALYMLSTRGRQTVKE	KDDTSFEDINGIRRP	FFSILQDMRNTIMAS	SMGIQLDQRIILLYM
AEKAVEMDPDDVNKN	RGRQTVKENKGTRIR	DDTSFEDINGIRRPK	FSILQDMRNTIMASK	DMDPELRGLAQALID
KAVEMDPDDVNKNTL	GRQTVKENKGTRIRF	DTSFEDINGIRRPKH	LQDMRNTIMASKTVG	MDPELRGLAQALIDQ
DAEKAVEMDPDDVNK	RQTVKENKGTRIRFK	TSFEDINGIRRPKHL	QDMRNTIMASKTVGT	DPELRGLAQALIDQK
NTLQARQQTVSALED	QTVKENKGTRIRFKD	SFEDINGIRRPKHLY	FYQSYLRRTQSMGIQ	PELRGLAQALIDQKV
TLQARQQTVSALEDK	GTRIRFKDDTSFEDI	FEDINGIRRPKHLYV	YQSYLRRTQSMGIQL	ELRGLAQALIDQKVK
GNVLDVNAIDIEEPS	TRIRFKDDTSFEDIN	RRPKHLYVSMPTAQS	QSYLRRTQSMGIQLD	LRGLAQALIDQKVKE
IILKALYMLSTRGRQ	RIRFKDDTSFEDING	RPKHLYVSMPTAQST	SYLRRTQSMGIQLDQ	RTQSMGIQLDQRIIL

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