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Multi Epitope Based Vaccine Design against *Capnocytophaga canimorsus* through Immunoinformatics Approaches

Levent Cavas*, Atakan Vatansever

Department of Chemistry, Faculty of Science, Dokuz Eylül University, Main Campus 35390, İzmir, Türkiye

*Corresponding author:levent.cavas@deu.edu.tr
Orcid No: https://orcid.org/0000-0003-2136-6928

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Abstract: Immunoinformatics has provided an important contribution to the acceleration of vaccine research. The *in silico* tools developed under immunoinformatics efficiently filter candidate vaccines and select the most appropriate ones for *in vitro* and *in vivo* studies. Multi epitope-based vaccine design against *Capnocytophaga canimorsus* infections through immunoinformatics approaches was proposed in the present investigation. Outer membrane protein (OMP) of *C. canimorsus* was used to develop peptide-based vaccines. IEDB tools are used in this research. The antigenic potential of *C. canimorsus* OMP was evaluated via VaxiJen v2.0 and the Overall Prediction for the Protective Antigen was found to be 0.6049. MHC-I and -II binding epitopes with maximum scores were found to be "QEIGKLKKY" for HLAB*44:03 and "FNAVQEIGK" for HLA-DRB5*01:01, respectively. ABCPrep analysis identified multiple epitopes. The maximum score of 0.91 was associated with the sequence "KNMRIGYVDMDFILEN". Discontinuous epitopes were also detected in this research with the maximum score observed for the regions A:L247, A:E248, A:Q250 and A:K251. The population coverage for the global population was calculated to be 96.45% for a defined set of epitopes. In conclusion, since the adoption of dogs and cats as pets has increased after COVID-19, there is a clear risk for *C. canimorsus* infections. The proposed peptide-based vaccines in this report may mitigate this risk on a global level.

Keywords: Antigenic potential; *Capnocytophaga canimorsus*; immunoinformatics; health biotechnology; vaccine

1 Introduction

Cats and dogs are important animals in human life. The domestication of dogs and cats has a significant effect on their evolutionary trajectory and their interactions with mankind (Ammerman, 2017). Moreover, this relationship is a crucial strategy for survival in a world where the influence of mankind has become dominant (Oltenacu, 2004). Archaeological evidence suggests that the domestication of dogs began at least 14,000 years ago. Additionally, the origin of unconscious domestication extends up to 35,000 years. Cats, on the other hand, were domesticated approximately 9,500 years ago (Galibert et al. 2011; Vigne et al. 2004). Not only did this co-evolution strengthen the bond between humans and animals, but it also gave benefits in tasks such as hunting and protection and had deep cultural importance in art, mythology, and literature (Schleidt and Shalter, 2003; De Tiège et al. 2021). However, this interaction also created a potential channel for the transmission of infections (Esposito et al. 2023). Animal caused diseases known as zoonoses, present a critical challenge to global public health because the infections in animals can pass to humans, which causes different kinds of diseases (Saklani et al. 2024). These diseases are caused by a wide variety of pathogens, such as bacteria, viruses, parasites, and fungi. Also, the transmission routes differ (Rees et al. 2021). Those diseases pass to humans through direct contact with animals, exposure to their saliva, bodily fluids, or excretions, or even indirectly, such as interaction with contaminated environments (Desvars-Larrive et al. 2024). Some of the most common zoonotic diseases are *Campylobacteriosis*, *Salmonellosis*, infection with Shiga toxin-producing *Escherichia coli* (STEC), *Yersiniosis*, *Listeriosis*, *Tularaemia*, *Echinococcosis*, Q fever, West Nile virus infection, *Brucellosis*, *Trichinellosis*, rabies, and congenital toxoplasmosis (EFSA, 2024). Although many zoonotic diseases are well understood, it is important to remember that understanding whether the zoonotic pathogens are related to their host is crucial because the change in host-pathogen interactions may result in the emergence of new diseases (Ko et al. 2009). These interactions are also related with recent trends, such as the rise in pet adoption during the COVID-19 pandemic (Pieracci et al. 2021; Hedman et al.

2021). As the COVID-19 pandemic started, the limitations imposed on people increased the level of stress and anxiety. Owning a pet was found to be the best choice for saving the well-being of humans, and a noteworthy increase in pet ownership was recorded during that period because pets provided companionship and social support during lockdown (Ho et al. 2021; Bowen et al. 2020). As people were stressed during isolation in the COVID-19 pandemic, pets gave a source of comfort and emotional connection (Dogbey et al. 2024). Even though the increase in human animal relationships has provided strong companionship, as it is mentioned earlier in the text, it has also poten-tially increased certain zoonotic risks (Powell et al. 2022). Increased contact with pets, like licking, scratching, and bit-ing could lead to a higher probability of transmission of com-mensal bacteria found in animal oral flora, such as *Capno-cytophaga* species. All seven species that constitute the genus *Capnocytophaga* are found in the oral cavities of humans and domestic animals (Shin et al, 2007). However, *C. canimorsus* is recognized as the most important species that causes human infections (Lion et al. 1996). This pathogen is part of the normal flora of the oral cavity of dogs and cats and is known to cause meningitis, sepsis, and other life-threatening diseases in human, especially those with compromised immune systems. *C. canimorsus* can be transmitted through dog bites or cat scratches (Butler, 2015). *C. canimorsus* also has high virulence and can evade the innate immune system. The bacteria have mechanisms such as catalase and cytotoxin production to evade phagocytosis, gliding motility to facilitate tissue invasion, and serum complement resistance (Shin et al. 2007). Symptoms that may be seen in affected individuals include sepsis, septic shock, multiorgan failure, purpura fulminans secondary to DIC and meningitis (Mader et al. 2020; Lee et al. 2020). The appearance of *C. canimorsus* has revealed the potential for zoonotic risk of the oral flora of animals and the need for clinician awareness of infections associated with animal bites (O'Riordan et al. 2021). Van Samkar et al. (2016) reported three cases of *C. canimorsus* meningitis, of which two, occurred in healthy individuals who were bitten by a dog. Even though the infections caused by *C. canimorsus* were firstly identified in 1976 by Bobo and Newton, who isolated it from a patient with septicemia following a dog bite, there is no commercially available vaccine for prevention (Butler, 2015; Popiel and Vinh, 2013) to the best of our knowledge. The complexity of the interaction between human and animal ecosystems creates a new call for preventing strategies from those dangers effec-tively, especially while facing zoonotic threats. Fortunately, thanks to the applications that have advanced immuno-informatics technologies, either the development of vaccines or the application of vaccine development immunotherapeutic treatments can be made efficiently without any time-consuming procedures. Vaccines targeting pathogens like *Neisseria meningitidis* and *Bacillus anthracis* are early examples of vaccines that were successfully developed using immunoinformatics and reverse vaccinology (Damas et al. 2022; Shamakhi and Kordbacheh, 2021). Additionally, the benefits of this approach were also explained in the report by Ortega-Tirado et al. (2020), as the facilities provided by immunoinformatics have made it possible to identify the immunogenic T-cell peptides of *Mycobacterium tuberculosis* for a new vaccine. Platforms like VaxiJel, UniProt, NCBI (National Center for Biotechnology Information), and IEDB (Immune Epitope Database) have became predominant in this field. These tools accelerate studies of biotechnology and immunology, thus allowing for more sensitive and effective results.

In this study, an immunoinformatics approach using epitope mapping, immunogenicity prediction, population coverage for MHC-I and II alleles, and detection of probable B and T-cell epitopes via IEDB.org tools was proposed for the development of linear and non-linear peptide-based vaccine candidates for *C. canimor-sus* outer membrane protein (OMP) coded A0A0B7H4B5.

2 Materials and Method

2.1 Data

The FASTA-encoded primary amino acid sequence of *C. canimorsus* OMP was obtained from the UniProt Knowledgebase (UniProtKB), specifically from the UniProt release version 2024 06. The protein sequence was accessed by the accession code of A0A0B7H4B5 (Coudert et al. 2023; Bateman et al. 2023).

2.2 Determination of vaccine potential via VaxiJen servers

The antigenic potential of OMP was evaluated using the VaxiJen v3.0 online tool (Dimitrov et al. 2020). This bioinformatics tool predicts antigenicity based on the physicochemical properties of protein sequences, as evidenced by papers containing data for novel immunogenic proteins tested on humans until March 2017 (Zaharieva et al. 2019). The sequences with a bacterial threshold of over 0.4 are classified as a probable antigen. Thus, the antigenic protein sequences of *C. canimorsus* OMP were analysed and ranked according to their antigenic scores, which demonstrates its importance for vaccine development.

2.3 MHC-I binding predictions

MHC-I binding prediction is one of the tools provided by IEDB.org (Andreatta and Nielsen, 2016; Lundegaard et al. 2008). In this analysis, the following criteria were used to predict MHC-I binding epitopes. NetMHCpan 4.1 (EL) was selected as the prediction method. Humans were the MHC source species. All alleles were selected with the 9 and 10-mer lengths as the default settings. The epitopes were ranked based on descending scores.

2.4 MHC-II binding predictions

To predict MHC class II binding epitopes, the IEDB prediction tool had been used, which provides a comprehensive platform for computational epitope identification. Specifically, the NetMHCIIpan 4.1 EL algorithm was preferred as the epitope prediction model.

NetMHCIIpan 4.1 EL predicts binding affinities of peptide sequences to multiple human leukocyte antigen (HLA) class II alleles by integrating peptide sequence motifs and structural information. The model takes into account the peptide length

variations and it was designed to improve prediction accuracy by integrating both eluted ligand and binding affinity data (Wang et al. 2008). The predictions were performed for a predefined set of HLA class II alleles.

All predicted epitopes were ranked based on their percentile scores, with lower percentile values indicating higher binding affinities. Only peptides falling within the top 2% rank were considered for further immunogenicity assessment, as suggested by previous benchmarking studies (Wang et al. 2010).

2.5 Prediction of T-cell epitopes and cleavage sites

Neural network-based prediction servers were used for the detection of cleavage sites (NetChop) and T-cell epitopes (NetCTL and NetCTLpan). NetChop is a predictor of proteasomal processing based upon a neural network (Nielsen et al. 2005). NetCTL and NetCTLpan are predictors of T cell epitopes along a protein sequence. It also employs a neural network architecture (Larsen et al. 2007). For the detection of the possible T-cell epitopes, all available twelve supertype alleles on NetCTL were used and the following filters were applied to each supertype: weight on C terminal cleavage: 0.15; weight on TAP transport efficiency: 0.05; threshold: 0.75. For the prediction of possible cleavage sites on the protein, the C-term 3.0 method was used, and a threshold value of 0.5 was used on NetChop.

2.6 B-cell epitope prediction on protein sequence

For B-cell epitope prediction, IEDB Analysis Resources (BepiPred 2.0, ElliPro and ABCpred tools) are preferred due to their effectiveness. Each tool uses specific algorithms and threshold values to identify sequences that can generate an immune response in our body. The IEDB Analysis Resource provides tools for predicting linear and conformational epitopes (Larsen et al. 2006). The BepiPred-2.0 server predicts B-cell epitopes from a protein sequence, using a Random Forest algorithm trained on epitopes and non-epitope amino acids determined from crystal structures (Jespersen et al. 2017). ElliPro predicts linear and discontinuous antibody epitopes based on a protein antigen's 3D structure (Ponomarenko et al. 2008). ABCpred server is based on machine learning techniques using fixed length patterns, to predict B-cell epitopes on an antigenic sequence (Saha and Raghava, 2006a). Together, these servers provide useful approaches to analyse antigenic features. ABCPred was employed using default settings, with a threshold value of 0.51 to obtain more precise predictions. ElliPro analysis was conducted by uploading the PDB file of *C. canimorsus* (fasta sequence of OMP was searched via alpha fold)(Jumper et al. 2021). Minimum score and maximum distance (Angstrom) parameters were used with default settings of 0.5 and 6, respectively.

2.7 Immunogenicity

The key property of *C. canimorsus* is its ability to produce an immunogenic response that results in the activation of T-cells. To identify the immunogenicity of OMP, ten distinct peptide sequences determined in MHC-I binding prediction (Table-1) were used by following the methodology outlined by Calis et al. (2013). In this stage of the research, selected immunogenic peptide sequences were analysed and ranked based on their immunogenicity score. These scores were calculated using the default settings where first, second, and C-terminus amino acids are masked.

2.8 Predictions of signal peptides within the protein sequence

Signal peptides play a critical role in the pathogenicity of microbial species and are of great importance for immune recognition by T and B cells (Owji et al. 2018). Signal peptide predictions were made on SignalP 6.0, server that predicts the presence of signal peptides and the location of their cleavage sites in proteins (Teufel et al. 2022). For the detection of signal peptides, the organism was chosen as others; the output format was set to long to view the signal peptides diagram, and the model mode was chosen as slow for an accurate result.

2.9 Population coverage

After predicting MHC class I and II binding epitopes for the OMP (Figure-1), a population coverage analysis was conducted on IEDB Population Coverage server to determine the percentage of individuals who could recognize specific epitope/HLA combinations for Türkiye and globally (Bui et al. 2006). While more than 380 MHC alleles were used for the global analyses, HLA-DQA1*01:01, HLA-DQA1*02:01, HLA-DQA1*03:01, HLA-DQA1*04:01, HLA-DQA1*05:01, HLA-DQA1*06:01, HLA-DQB1*02:01, HLA-DQB1*03:01, HLA-DQB1*04:01, HLA-DQB1*05:01, HLA-DQB1*06:09, HLA-DRB1*01:01, HLA DRB1*03:01, and HLA-DRB1*04:01 alleles were used for the population coverage of Türkiye. Two analyses were performed separately for combined MHC classes (MHC-I and II), using the critical sixteen immunogenic epitopes (ten for MHC-I and six for MHC-II). The following filters were applied for IEDB Population Coverage of Türkiye: number of epitopes: 16; query by: area country ethnicity; calculation options: class I and II combined; selected area or population: Türkiye. For global, the number of epitopes was set as 16; query by: area country ethnicity; calculation options: class I and II combined; selected area or population: world.

2.10 Kolaskar and Tongaonkar antigenity

The Kolascar and Tongaonkar Antigenity method is a semi-empirical approach preferred to predict antigenic determinants, or B-cell epitopes, on protein antigens. This method uses the physicochemical properties of amino acid residues, such as hydrophilicity, accessibility, and flexibility, along with their frequencies of occurrence in experimentally determined epitopic regions. By analyzing these parameters, the method identifies potential antigenic sites within a protein sequence. In the present study, the Kolaskar and Tongaonkar Antigenicity method to was applied OMP to identify immunogenic regions (Kolaskar and Tongaonkar, 1990).

2.11 Prediction of antigenity of protein sequence

The allergenicity of protein sequences was predicted using two bioinformatics tools, namely AlgPred and Allermatch-TM. Algpred allows prediction of allergens based on similarity of known epitope with any region of the protein, and this tool integrates motif-based detection, SVM-based classifica-

tion, and epitope mapping to predict allergenic proteins (Saha and Raghava, 2006b). Input sequences were analyzed for the presence of IgE-binding motifs and allergen representative peptides. AllermatchTM uses a FASTA-based search to compare protein sequences against known allergen databases (AllergenOnline)(Fiers et al. 2004).

3 Results

3.1 Determination of vaccine potential of *C. canimorsus* OMP via VaxiJen v2.0 and 3.0.

The overall predicted value for the Protective Antigen was found to be 0.6049, when the VaxiJen 2.0 version was used. On the other hand, VaxiJen 3.0 version gives more accurate output by stating "Probable IMMUNOGEN with a probability of 100%.".

3.2 MHC-I binding predictions

OMP was checked for MHC-I binding epitope predictions. The results related to the epitope analysis for MHC-I are presented in Table-1. According to MHC-I analysis guidelines, the peptides found were ranked based on their scores. The linear peptides with the best scores greater than 0.9 are shown in Table-1. All available alleles were selected under the default settings in IEDB MHC-I epitope prediction. According to the results, the maximum score was associated with "QEIGKLKKY". This sequence was also found for HLA-B*44:03. The allele frequency of HLA-B*44:03 was also checked via the allele frequencies database (Gonzalez-Galarza et al. 2020). The people who carry HLA-B*44:03 alleles are shown in Figure-1. Although Figure-1 shows the worldwide distribution of the HLA-B*44:03 alleles, the maximum percentage of individuals with the allele is 23.2, found in India East UCBB. Allele Frequency was also found to be 0.125.

Table 1 The MHC-I epitope prediction results for *C. canimorsus* OMP.

Allele	Sequence Number	Start	End	Length	Peptide	Score	Peptide Rank
HLA-B*44:03	3	15	23	9	QEIGKLKKY	0.994842	0.01
HLA-B*44:02	3	15	23	9	QEIGKLKKY	0.993719	0.01
HLA-A*68:01	4	15	23	9	EVVDEKAQR	0.981630	0.01
HLA-B*15:01	1	3	11	9	KQVIHSVVF	0.968909	0.01
HLA-A*11:01	1	15	23	9	ATTGLFAQK	0.961922	0.01
HLA-A*03:01	3	45	53	9	RLILRVINK	0.957827	0.01
HLA-B*15:01	1	21	29	9	AQKNMRIGY	0.948779	0.01
HLA-B*35:01	1	34	42	9	FILENVEEY	0.922386	0.03
HLA-B*44:03	1	40	49	10	EEYKIASAQF	0.906822	0.04
HLA-A*02:06	2	34	42	9	AILEHNLRV	0.900030	0.04

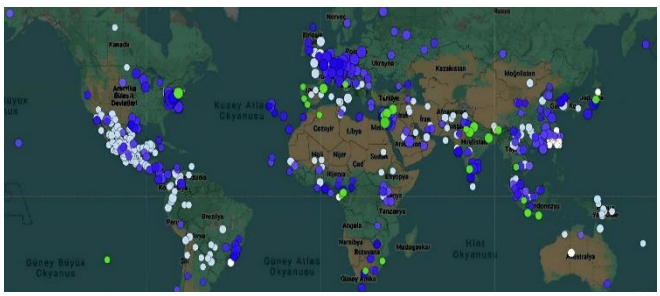

Fig. 1 Worldwide allele frequency of HLA-B*44:03 via Allele Frequencies.

3.3 MHC-II Epitope Binding Prediction Results

MHC-II Epitope binding predictions were carried out via IEDB.org. The best six results with scores higher than 0.7 are shown in Table-2. The results showed that the maximum score was found for "FNAVQEIGK" with respect to the allele "HLA-DRB5*01:01". This result is for HLA-DR in default settings. No data were found for HLA-DRB5*01:01 in the allele frequency database. However, the data was found for HLA-DRB1*07:01. This allele was very common in Central African Republic (Aka Pygmy) with 42.9% of individuals having the allele and its frequency being 0.2440. Figure-2 demonstrates the worldwide distribution of HLADRB1*07:01 alleles by using different colored markers, to distinguish various HLA alleles across different geographic regions.

Table 2 MHC-II epitope binding prediction results for OMP.

Allele	Start	End	Length	Core Sequence	Peptide Sequence	Score	%Rank
HLA-DRB5*01:01	128	142	15	FNAVQEIGK	DQVFNAVQEIGKLKK	0.8243	0.10
HLA-DRB1*07:01	38	52	15	YKIASAQFA	NVEEYKIASAQFAQQ	0.8171	0.43
HLA-DRB1*07:01	39	53	15	YKIASAQFA	VEEYKIASAQFAQQV	0.8066	0.45
HLA-DRB1*03:01	192	206	15	EVVDEKAQR	YDFEVVDEKAQRKAE	0.7268	1.20
HLA-DRB1*07:01	37	51	15	YKIASAQFA	ENVEEYKIASAQFAQ	0.7165	0.92
HLA-DRB1*15:01	97	111	15	LRVYQQEKF	EHNLRVYQQEKFGAE	0.7097	0.88

Table 3 T-cell epitopes with their prediction score.

Supertypes	#	Peptide Sequence	Predicted MHC Binding Affinity	Rescale Binding Affinity	C Terminal Cleavage Affinity	Transport Efficiency	Predictions Score
A1	149	KSDVSMLYS	0.3685	2.5024	0.0123	2.4410	2.3822
B44	135	QEIGKLKKY	0.2469	1.7072	0.9356	2.9180	1.9934
A3	15	ATTGLFAQK	0.6121	1.8229	0.9243	0.5990	1.9914
B27	228	QRLQEREQK	0.3227	1.5939	0.5686	0.6530	1.7118
B62	3	KQVIHSVVF	0.5345	1.3668	0.8092	2.8960	1.6330
A26	34	FILENVEEY	0.4460	1.2943	0.9988	2.8370	1.5859
B8	1	MKKQVIHSV	0.2751	1.3318	0.9660	0.4070	1.4971
B39	6	IHSVVFLLL	8.9900	1.2662	0.9987	1.0280	1.4674
A24	41	EYKIASAQF	0.8296	1.1351	0.9940	2.4260	1.4055
A2	34	AILEHNLRV	0.4226	1.0114	0.9941	0.6140	1.1912
B7	87	KDREQEIAI	0.2624	0.7277	0.1500	0.3920	0.7698
B58	139	KLKKYDFIF	0.1410	0.4877	0.9946	2.6290	0.7683

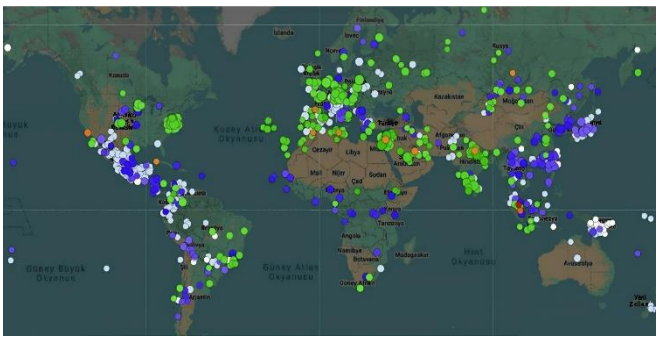

Fig.2. Worldwide allele frequency of HLA-DRB1*07:01 via Allele Frequencies

3.4 Prediction of T-cell epitope and cleavage sites

The data given in Table-3 demonstrate the predicted T-cell epitopes and their respective prediction scores for each supertype in detail. These scores contain multiple parameters such as MHC binding affinity, rescale binding affinity, C-terminal cleavage affinity, and TAP transport efficiency, which can be understood well from the overall prediction score. The data show the variability in peptide sequences and their corresponding immunogenic potential for each supertype. Supertypes with a high prediction score are likely to be recognized effectively by T-cells. All the peptide sequences for each supertype are ordered according to their prediction score, and the peptides with the highest scores for each supertype are placed in Table-3.

Cleavage predictions obtained from NetChop provide critical information about the immunogenic potential of the protein and its ability to be a candidate for vaccine development. Figure-3 demonstrates the predicted proteasomal cleavage sites of the OMP. While the green regions remain under observation, further analysis is conducted to determine their impact. The prediction score for the cleavage above the threshold (score $\geq$ 0.5) represents positive cleavage predictions, while pink regions, where the prediction score is below the threshold, suggest lower probabilities. The distribution of cleavage sites spread along the protein sequence with several high-scoring regions.

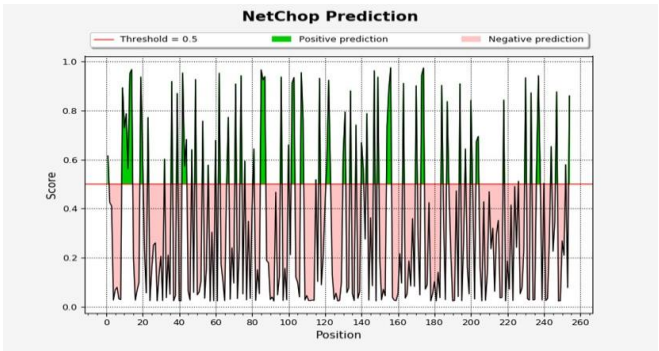

Fig. 3 Possible proteasomal cleavage sites of *C. canimorsus* OMP.

3.5 B-cell epitope predictions

For the prediction of potential B-cell epitopes, three immunoinformatics tools (BepiPred 2.0, ElliPro and

ABCpred) were used for the analysis of *C. canimorsus* OMP. Table-4 displays different probable epitope regions identified using various tools. Amino acid sequences with the prediction score over the threshold value were considered as potential B-cell epitopes. ABCPrep analysis identified multiple epitopes, with the highest-scoring peptide as KNMRIGYVDM-DFILEN (0.91). Additionally, epitopes with high scores were: KESAEDRNKSMAELLK (0.86), EIAILEHNLRVY-QQEK (0.84), and PELIKDREQEIAILEH (0.81). ElliPro analysis of the OMP of *C. canimorsus* revealed valuable information about predicted B cell epitopes. Two linear epitopes were identified, with lengths of 24 and 15 amino acids, respectively. The first epitope with the residues 1-24 (MKKQVIHSVVFLLLATTGLFAQKN) exhibits a high score of 0.791, indicating strong antigenicity. The second epitope consisting of residues 172-186 (NKKESAEDRN-KSMAE) has a lower score of 0.583. BepiPred identified two potential longer linear B-cell epitopes, 82 and 72 residues long, and four potential shorter linear B-cell epitopes, 5, 1, 2, and 11 residues long, with specific sequences. The yellow area in Figure-4 demonstrates possible epitope regions of the amino acid sequences with the prediction scores over 0.5. Green regions, on the other hand, belong to the peptides with the lowest prediction scores.

Table 4 Linear B-cell epitope predictions and corresponding scores of *C. canimorsus* OMP using BepiPred, ABCPred, and ElliPro.

B-Cell Epitope Prediction Tools	Probable Linear B-Cell Epitope Sequences and Scores
BepiPred 2.0	QHNLS
	L
	RVINKKESAEDRNKSMAELLKENYDFEVVDE KAQRKAEIEQARQQR AQEREKQREAARQQRLQEREQKKKEAEERKK KLEEQ
	QD
	AQQVEQWEAEIEKRKTKIEAEKNKLEAEKPLL TPELIK DREQEIAILEHNLRVYQQEKFGAENGEYVKQK FMLAKP
	ENVEEYKIASA
ABCPred	KNMRIGYVDMDFILEN (0.91)
	KESAEDRNKSMAELLK (0.86)
	EIAILEHNLRVYQQEK (0.84)
	PELIKDREQEIAILEH (0.81)
	AEIEKRKTKIEAEKNK (0.81)
	EQKKKEAEERKKKLEE (0.81)
	DFIFEKSDVSMLYSNN (0.79)
	LKENYDFEVVDEKAQR (0.77)
	EAEKNKLEAEKPLLTP (0.74)
	KAEIEQARQQRAQERE (0.73)
	GAENGEYVKQKFMLAK (0.72)
	NLRVYQQEKFGAENGE (0.70)
	GKLKKYDFIFEKSDVS (0.70)
	LAKPIQDQVFNAVQEI (0.70)
ElliPro	MKKQVIHSVVFLLLATTGLFAQKN (0.79)
	NKKESAEDRNKSMAE (0.58)

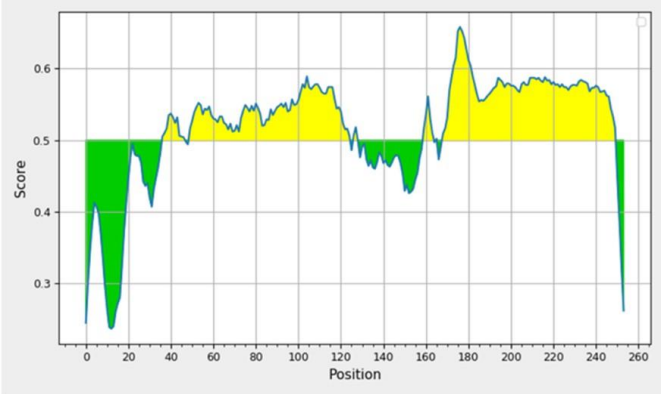

Fig. 4 BepiPred positions of B-cell epitopes on OMP.

Seven discontinuous (conformational) epitopes were predicted with scores ranging from 0.975 (highest) to 0.574 (lowest) via Elliport (Table-5). The top-ranked discontinuous epitope consists of four residues (L247, E248, Q250, K251) with a score of 0.975. The most extensive epitope, consisting of 36 residues (A66–A101), has a score of 0.768. 3D structures for either continuous (linear) or discontinuous epitopes are shown in Figure-5 and Figure-6.

Table 5 Discontinuous B-cell epitopes of OMP predicted via ElliPro.

#	Residues	# Residues	Score
1	A:L247, A:E248, A:Q250, A:K251	4	0.975
2	A:Q235, A:K236, A:E239, A:A240, A:E241, A:R243, A:K244	7	0.897
3	A:Q4, A:V5, A:I6, A:H7, A:S8, A:V9, A:V10, A:F11,A:L12, A:L13, A:L14, A:A15, A:T16, A:T17,	19	0.795
4	A:G18, A:L19, A:F20, A:A21, A:Q22,A:K66, A:I67, A:E68, A:A69, A:E70, A:K71, A:N72, A:K73, A:L74, A:E75,A:A76, A:E77, A:K78, A:P79, A:L80, A:L81, A:T82, A:P83, A:E84,A:L85, A:I86, A:K87, A:D88, A:R89, A:E90, A:Q91, A:E92, A:I93,A:A94, A:I95, A:L96, A:E97, A:H98, A:N99, A:R101, A:V102	36	0.768
5	A:A215, A:R218, A:E219, A:K220, A:E223, A:A224, A:A225, A:R226,A:Q227, A:Q228, A:R229, A:L230, A:Q231, A:R233	14	0.683
6	A:E136, A:K173, A:K174, A:S176, A:A177, A:E178, A:D179,A:R180, A:N181, A:K182, A:S183, A:M184, A:A185, A:E186	14	0.598
7	A:A58, A:E59, A:I60, A:E61, A:K62, A:R63, A:K64, A:T65, A:L100	9	0.574

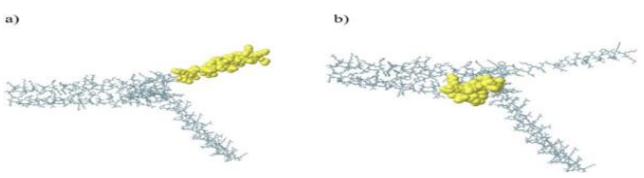

Fig. 5 3D structure of the predicted linear B-cell epitope regions for (a) the peptide sequence "MKKQVIHSVVFLLLATTGLFAQKN" (b) the peptide sequence "NKKESAEDRNKSMAE" in Table-4).

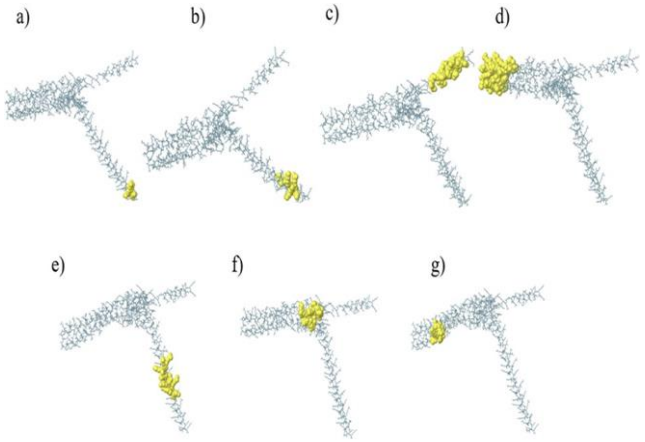

Fig. 6 3D structure of the location of predicted discontinuous B-cell epitopes of (a) sequence#1,(b) sequence#2, (c) sequence#3, (d) sequence#4, (e) sequence#5, (f) sequence#6, (g) sequence#7 via Ellipro.

3.6 Immunogenicity

The results for *C. canimorsus* OMP are given in Table-6. Those results demonstrate the probabilities of the different peptide sequences found in the OMP in generating an immune response when it enters the body. The peptide sequence "FILENVEEY" has the highest score (0.27271) meaning that it has a strong potential to generate an immune response. Similarly, peptides like RLILRVINK (0.22984) and AILEHNLRV (0.14344) demonstrate moderate immunogenic potential. Peptides with negative scores such as QEIGKLKKY (-0.45114) and EEYKIASAQF (-0.24089) have a lower probability of generating a significant immune response. The peptides with zero/near-zero scores, AQKNMRIGY (-0.06667), indicate a neutral activity, means that the peptide is unlikely to generate a strong immune response.

Table 6 Immunogenicity of MHC-I binding epitopes of *C. canimorsus* OMP.

Peptide	Length	Score
FILENVEEY	9	0.27271
RLILRVINK	9	0.22984
AILEHNLRV	9	0.14344
ATTGLFAQK	9	0.11144
KQVIHSVVF	9	0.08205
AQKNMRIGY	9	-0.06667
EVVDEKAQR	9	-0.10444
EEYKIASAQF	10	-0.24089
QEIGKLKKY	9	-0.45114
QEIGKLKKY	9	-0.45114

3.7 Prediction of signal peptides within protein sequence

Proteins containing signal peptides are commonly involved in virulence mechanisms and the SignalP 6.0 prediction diagram for the *C. canimorsus* OMP demonstrates the pres-ence of a Sec/SPI signal peptide (Antelmann et al. 2001). The initial segment of the signal peptide (positions 1–5) is high-lighted with a high probability for the N-region (Figure-7).

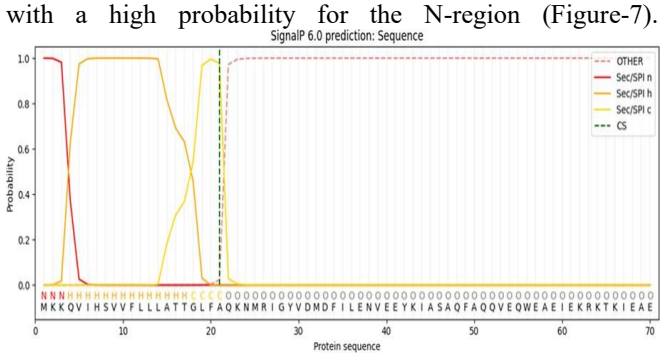

Fig. 7 Possible signal peptides on *C. canimorsus* OMP

This positively charged region plays a critical role in directing the protein to the secretion machinery. Following the N-region, the graph indicates a highly hydrophobic segment (positions 5-15), characteristic of the H-region. This hydrophobic side anchors the signal peptide in the membrane during translocation. The cleavage site was marked at positions 21-22, and the probability of this prediction is 0.9767. Beyond those positions, the probability passes to the "Other" category. While the probability of the sequence not having a signal peptide, categorized as 'Other,' is 0.0005, the likelihood for a classical signal peptide, categorized as 'Sec/SPI,' is 0.9986. The probability for lipoprotein signal peptide (Sec/SPII) is 0.0003. The probability of the other three types (Tat/SPI, Tat/SPII and Sec/SPIII) is 0.0002.

3.8 Population coverage

The results demonstrated that 94.57% of the population of Türkiye is covered by at least one epitope/HLA combination for combined MHC class I and II epitopes which means that the selected epitopes and HLA combinations affect large population coverage. On average, an individual recognizes approximately 25.2 epitope/HLA combinations. The minimum number of epitope/HLA combinations required per individual to achieve 90% population coverage (PC90) is 15.

Due to the absence of available allele frequency data for HLA-DRB5*01:01 for MHC-II, the population coverage analysis was conducted without including this allele. Despite this limitation, the global population coverage was calculated to be 96.45% which indicates that nearly the whole global population can recognize at least one epitope/HLA combination. On average, an individual can recognize 22.97 epitope/HLA combinations. Additionally, the PC90 value (the minimum number of epitope/HLA combinations required to cover 90% of the population) was found to be 13.34.

3.9 Kolaskar & Tongaonkar antigenity determination

Several peptide regions with high antigenicity scores (Table-7) have been identified in the Kolaskar and Tongaonkar antigenicity analysis for the OMP of *C. canimorsus*. The table highlights residues with scores exceeding 1.005. Among the top candidates, sequences such as "VFVLLLA" (1.239), "SVVFLLL" (1.231), and "VIHSVVF" (1.216) exhibit the highest scores.

The graphical representation (Figure-8) shows a distinction between high antigenicity regions (yellow) and less antigenic

areas (green). Also, regions with residues from positions 9–15 and 165–171 demonstrate high antigenicity.

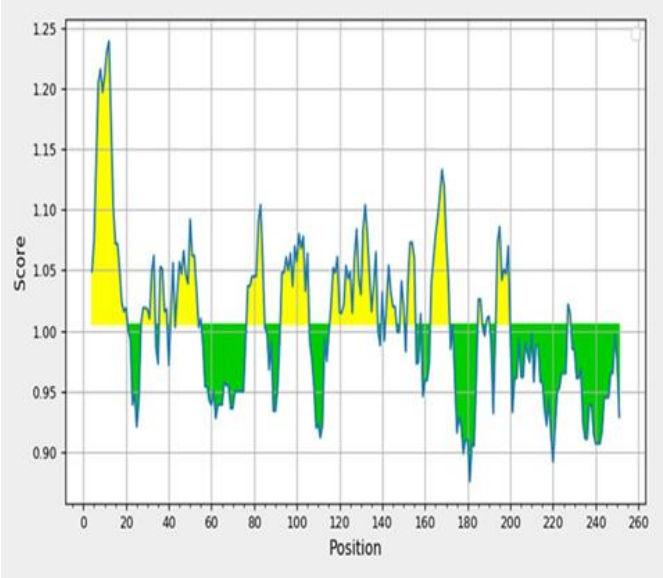

Fig. 8 Kolaskar & Tongaonkar antigenicity graphic of OMP.

Table 7 Predicted antigenic residues of OMP via Kolaskar & Tongaonkar Antigenicity.

Position	Residue	Start	End	Peptide	Score
12	L	9	15	VVFLLLA	1.239
11	F	8	14	SVVFLLL	1.231
8	S	5	11	VIHSVVF	1.216
10	V	7	13	HSVVFLL	1.211
7	H	4	10	QVIHSVV	1.205
9	V	6	12	IHSVVFL	1.197
13	L	10	16	VFLLLAT	1.171
6	I	3	9	KQVIHSV	1.140
168	L	165	171	RLILRVI	1.133
169	R	166	172	LILRVIN	1.119
167	I	164	170	SRLILRV	1.113
83	P	80	86	LLTPELI	1.104
132	N	129	135	QVFNAVQ	1.104
14	L	11	17	FLLLATT	1.103
166	L	163	169	LSRLILR	1.094
50	A	47	53	AQFAQQV	1.092
82	T	79	85	PLLTPEL	1.091
195	E	192	198	YDFEVVD	1.086
128	D	125	131	PIQDQVF	1.084
131	F	128	134	DQVFNAV	1.083
101	R	98	104	HNLRVYQ	1.080
133	A	130	136	VFNAVQE	1.080
165	R	162	168	NLSRLIL	1.080

3.10 Allergenity

Allergenicity of the protein sequences was analysed by using two different tools. AlgPred identified 85.64% (positive predictive value) and 67.96% (negative predictive value) of the sequences as potential allergens with a score of 1.1542393, based on the presence of IgE binding epitopes and motif analysis.

In AllerMatch, the sequence of OMP shows significant matches to known allergens, as indicated by the E-values (0.058) and the close identity percentages (20–26%). These results suggest that the sequence is likely allergenic, particularly due to its similarity to known allergens such as paramyosin from *Anisakis simplex* (*Ani s 2*) and *Anisakis pegreffii*.

4 Discussion

This investigation developed a multiepitope-based vaccine design against *C. canimorsus* through immunoinformatic approaches. The diseases caused by contact with pets such as a bite or lick, negatively affect patients' general health conditions who do not take any treatment (Beauruelle et al. 2022). It is well demonstrated that nearly the whole global population can develop immunogenicity against the epitope/HLA combination for *C. canimorsus* OMP. In a review written by Zajkowska et al. (2016), the danger of underestimating *C. canimorsus* infection is very well explained. Moreover, the authors mentioned that this bacterium could pose a significant risk for patients with asplenia, cirrhosis, or alcohol abuse. Since these conditions are not only valid for dog bites, but also for cat bites, vaccination might be necessary for some patients. Due to the conditions of the COVID-19 pandemic, the prevalence of pet ownership has increased all around the world (Ho et al. 2021). Therefore, even if the negative effects have not so far been published, possible peptide-based vaccines may be required for pet owners in the near future due to the increased number of dogs and cats as pets. According to a very recent case study reported by Yang et al. (2021), *Capnocytophaga*-based blebitis was reported in a case involving close contact with a dog including face licking (Yang et al. 2021). *C. canimorsus*-based endocarditis (Sandoe, 2004), meningitis and bacteraemia (Galles et al. 2020; Hannon et al. 2020, and purpura fulminans (Parisi and Pihán, 2023) were also discussed in the scientific literature. Due to the lack of any available specific vaccine for the bacterium, the latter disorders can be observed in people who have pets (Butler, 2015). Nowadays, immunoinformatics provides important contributions to the development of new and efficient vaccines (Kushwaha et al. 2024). This approach is suitable for the principles of reverse vaccinology and immunoinformatics, and it enables the identification of immunogenic targets by using computational tools (Masum et al. 2024). Such methodologies have a great potential to accelerate vaccine design. The ability to predict which proteins are most likely to generate immune responses allows researchers to increase productivity in discovering vaccines for various pathogenic species through the use of these tools. The results showed that *C. canimorsus* OMPs have demonstrated significant potential as vaccine and diagnostic candidates due to their immunogenic property. The epitope-rich regions of OMP are critical for their interaction with host immune cells, because those regions are the ones by which the bacterium is detected and recognized by T and B cells (Li and Wu, 2021). Krishnan et al. (2021) have also used similar tools for the

development of T cell multi epitope dengue peptide vaccine. They studied non structural proteome. The authors also reported that the epitopes HTLWSNGVL and FTTNIWLKL are the most stable among the epitopes studied in their paper. This approach showed that the probability of the interaction between immune cells, like T and B cells, with epitopes has increased, leads to a stronger immune response. In this study, we have highlighted the structural and antigenic properties of *C. canimorsus* OMP. In this investigation, different *Capnocytophaga* genus members were also compared. The sequence of the OMPs of *C. canimorsus* (A0AAD0EAI0) was compared with those of *C. gingivalis* (A0A250FNB1) and *C. cynodegmi* (A0A250E913). Multiple sequence alignment analysis (Figure-9) revealed that different species within the genus *Capnocytophaga* may show a different amino acid sequence trend.

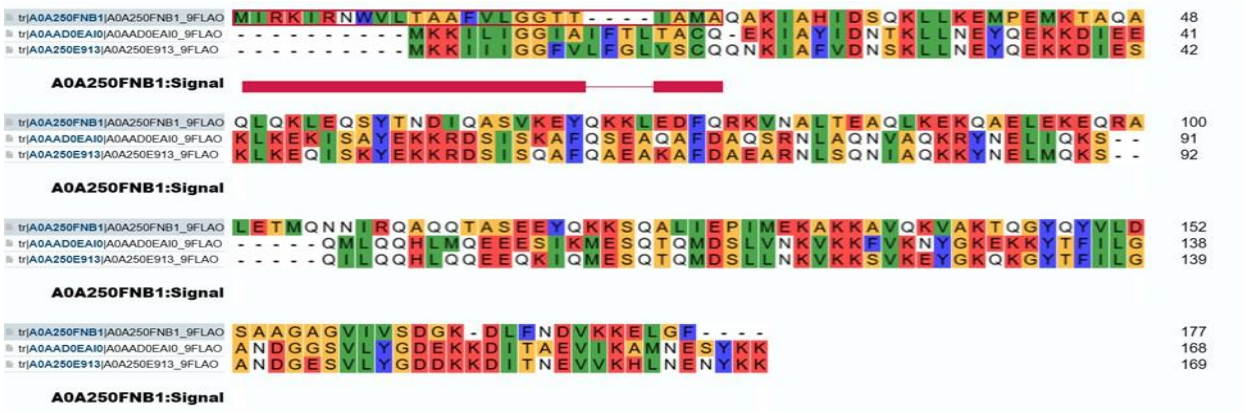

Fig. 9 Multiple sequence alignment analysis of *C. canimorsus* (A0AAD0EAI0), *C.gingivalis* (A0A250FNB1) and *C. cynodegmi* (A0A250E913).

Therefore, the vaccine developed based on *C. canimorsus* OMP may not show enough protection due to sequence differences. The antigenic potential of the *C. canimorsus* OMP was evaluated by using the VaxiJen v2.0, and 3.0 bioinformatics platform (Dimitrov et al. 2020). For this study, the protein sequences of *C. canimorsus* OMPs were input into the VaxiJen system. The antigenicity of the proteins has been calculated using the algorithm to analyze factors such as hydrophobicity, molecular weight, and amino acid composition. Based on this analysis, a ranked list of potential antigens has been created and the antigens that contain proteins with the highest scores were accepted as the most likely candidates for vaccine development. Notably, VaxiJen 2.0 showed significantly different results for three species. Although overall prediction of the protective antigen value was found to be 0.3977 for *C. gingivalis*, it was estimated to be 0.2776 and 0.3030 for *C. canimorsus* and *C. cynodegmi*, respectively. These results clearly showed that the selection of the correct sequence for immunoinformatics analysis is of great importance. The findings of our study demonstrated that the immunoinformatics approaches for designing multiepitope-based vaccines could be applied for *C. canimorsus* infections after *in vitro* and *in vivo* experiments. Joluvet-Gougeon et al. (2007) recommended that Imipenem /cilastatin, clindamycin, or beta-lactamase inhibitor combin-ations be used for antimicrobial therapy strategy against *Capnocytophaga* infections. Sandoe (2024) reviewed 12 cases of endocarditis caused by *C. canimorsus*, and the review underlined the importance of penicillins for the treatment of *C. canimorsus* infections. However, three cases were reported as deaths. Although most of the patients gave positive results after penicillin treatment, nonspecific symptoms of *C. canimorsus* can cause serious conditions such as septic shock and multiorgan failure (Meyer et al. 2021). According to a recent study on *Capnocytophaga* spp., minimum inhibitory concentrations (MIC) of various antibiotics and resistance genes were studied for 6 species under genus *Capnocytophaga* (Umeda et al. 2024). The researcher reported very low MIC values for imipenem and amoxicillin/clavulanic acid. It is very important to note that Umeda et al. (2024) reported a mutation in the quinolone resistance-determining region of gyrA .

As mentioned in the present study, there is a significant sequence variability in the OMPs among *Capnocytophaga* species which makes it difficult to identify vaccine targets. The variability observed in the multiple sequence alignment demonstrates the importance of selecting appropriate antigenic sequences for immunoinformatic analysis and vaccine development. Several factors are related to the absence of a traditionally developed vaccine against *C. canimorsus* infections. Primarily, the relatively low incidence of serious infections, reported at 0.67 infections per million, poses a significant challenge (Van Dam and Jansz, 2011). Furthermore, the genetic diversity of these pathogens, insufficient knowledge of host-pathogen interactions, lack of suitable cell lines, and absence of reliable animal models further block the traditional vaccine development efforts (Sunita et al. 2020). The absence of a specific vaccine against *C. canimorsus* can be attributed to several factors such as immunoinformatics-based studies have shown positive results for other related bacterial pathogens. For example, a multi-epitope vaccine that targets *C. gingivalis*, one of the bacterial species that is closely related within the same genus, was recently developed using similar methods (Repac et al. 2021). This shows the benefit of using computational tools to accelerate vaccine design. Nevertheless, the antigenic variations between *C. gingivalis* and *C. canimorsus* show that cross-protection will be insufficient due to the difference in the peptide sequences; and this demonstrates the critical need for species-specific vaccine development. A previous study

also demonstrates that *C. canimorsus* OMP have relatively lower antigenic scores compared to other *Capnocytophaga* species. This explains the need for more research to improve vaccine efficacy. While immunoinformatics provides a strong infrastructure for the identification of vaccine candidates, there are several steps that should be taken to translate these findings into practical applications. *In vitro* and *in vivo* studies of predicted epitopes have great importance because experimental data help to confirm their immunogenicity. Furthermore, it is also crucial to understand the effectiveness and safety of the identified epitopes and confirm that those epitopes can generate a strong immune response without causing negative effects. Furthermore, verification of the epitopes that can generate a strong immune response without negative effects like immunopathology, autoimmunity, or hypersensitivity reactions is crucial for understanding their efficacy and safety (Cusick et al. 2012). While improving the stability, immunogenicity, and targeted delivery of the vaccine can-didates, researchers should carefully consider the combination of adjuvants and delivery systems to apply these findings. Adjuvants such as aluminium salts or saponins can increase the host immune response and activate both humoral and cellular immunity (Wang, 2021). Furthermore, designing appropriate vaccine delivery systems, such as nanoparticles, liposomes, or viral vectors, can protect the epitopes from degradation and maintain their release (Pati et al. 2018). These systems enhance their overall effectiveness. To determine the safety and immunogenicity of the vaccines, clinical tests are useful as they provide direct results on their effects on humans (Gebre et al. 2021; Vinusha and Girish, 2024). In addition, these tests helpful in determining the cross protective potential of vaccines against different pathogens. This is because different pathogens have different anti-genicity and this difference may affect the efficacy of the vaccine (Warimwe et al. 2021). These varieties can also be easily detected and would be very useful in aiding the design of multi-epitope vaccines. This can be accomplished by using bioinformatics tools such as structural modeling and mole-cular docking, along with experimental data (Yurina and Adianingsih, 2022). Furthermore, to overcome the common issues that may be encountered when transferring from small scale to large scale manufacturing, it is important to focus on strong quality control measures and production plans for vaccine candidates that move from research to market (Buckland et al. 2024). During the manufacturing process, some of the important procedures are optimizing the protocols, production costs and following the guidelines of Good Manufacturing Practices (GMP) for the vaccines to be used in clinics and by patients (Silva et al. 2022).

5 Conclusion

This investigation shows the possibility that *C. canimorsus* OMPs can be used as vaccine candidates, and it also underlines the role of immunoinformatics in vaccine design. Although there are several limitations such as antigenic variability and the rarity of *C. canimorsus* in vaccine literature, our findings offer a starting point for further investigation. Applying computational predictions with experimental data and new vaccine delivery systems, considering the increasing demand for preventive strategies, especially due to the rise in pet ownership and the resulting zoonotic diseases, would be useful.

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CRediT authorship contribution statement

Levent Cavas: Conceptualization, Investigation, Methodology, Resources, Supervision, Validation, Writing – review and editing.

Atakan Vatansever: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data generated and analyzed during this study are included in this published article.

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