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Comparative *in Silico* Analysis of Heme Oxygenase Protein Sequences from Geographically Diverse Isolates of *Leptospira interrogans*

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Abstract: Leptospirosis is a resurgent infectious disease that has a vital impact on public health. It is caused by the widely dispersed zoonotic bacterium *Leptospira interrogans*. The enzyme heme oxygenase which is essential for both iron uptake and heme breakdown, is crucial to the pathogen's ability to survive and spread throughout the host. This study conducts a comparative *in silico* analysis of heme oxygenase protein from geographically diverse isolates of *Leptospira interrogans* to understand structural, functional, and evolutionary variations. Multiple sequence alignment and phylogenetic analysis of heme oxygenase proteins from geographically diverse regions spanning 15 countries revealed its mostly conserved nature with few unique amino acid substitutions. Among the substitutions few were observed to be non-conservative and charge altering amino acid substitutions like Phe84Ile, Asp102Asn, Asp181Asn, and Lys105Gln. These substitutions may affect enzyme stability, substrate binding affinity and active site conformation. A particular hotspot of mutation at Lys105Gln probably due adaptive evolution was also identified. Furthermore, evolutionary analysis revealed that heme oxygenase protein is mostly conserved as the isolates segregated into two major clades with lesser geographical separation. It also indicated common ancestry among the heme oxygenase proteins. This study highlights the potential conserved nature of heme oxygenase and can be treated as a target for therapeutics. However, further structural validation and experimental evidence is required to validate such potential.

Keywords: Heme oxygenase, Leptospirosis, *Leptospira interrogans*, Multiple sequence alignment, Amino acid substitutions, Phylogenetic analysis

Introduction

Leptospira interrogans is a pathogenic spirochete that responsible for leptospirosis, a well-known worldwide zoonotic disease [1]. Within the genus of *Leptospira*, pathogenic species include *Leptospira interrogans* and *Leptospira borgpetersenii*, whereas *Leptospira biflexa* comprises intermediate and non-pathogenic species [2]. The pathogenic groups have varying capacities to cause disease and adapt to changes in the environment. These are spiral shaped as well

as motile and flourish well in water and moist soil. In order to identify these microorganisms, a wide range of approaches such as microscopy, culture-based methods, and molecular sequencing have been used [3].
Leptospirosis is an emerging health issue, especially in countries with tropical and subtropical climates. More than a million cases are reported every year, and death rates can reach up to 20 percent in severe infections [4]. Transmission primarily occurs through direct contact with contaminated water or soil or with urine of infected animals, particularly rodents. People engaged in professions like farming, sewage handling, and veterinary work are at greater risk and are more vulnerable to infections [5]. The disease is characterized by symptoms ranging from mild to severe fever along with complications such as jaundice, kidney failure in renal patients, pulmonary haemorrhage, meningitis, and Weil's disease [6].

Pathogenicity of *Leptospira interrogans* has been ascribed to several virulence factors important for adhesion, invasion, and immune evasion or persistence in the host environment [7]. Among these, the heme oxygenase protein encoded by the *hemO* gene is a critical virulence determinant associated with iron acquisition and essential in determining pathogenicity [8]. Iron is an important trace element for pathogenic bacteria and plays an essential role in metabolic processes like the tricarboxylic acid cycle, electron transport system, and amino acid biosynthesis. Because of the limited amount of iron available within the host environment, bacteria have evolved specialized mechanisms for iron acquisition, closely associated with their virulence [9-10].

Leptospira interrogans uses iron through enzymatic degradation of heme present in haemoglobin, as iron is a limiting factor within the host [11]. Heme oxygenase catalyzes the oxidative degradation of heme to biliverdin, free iron, and carbon monoxide in a sequential monooxygenation reaction by consuming molecular O_2 and reducing equivalents [12]. The catalytic region of heme oxygenase is highly consistent in pathogenic strains of *Leptospira* genus, even though variable regions are also witnessed. This protein is absent or non-functional in non-pathogenic species, supporting its role in virulence [13]. Predominantly, heme binding by enzymes occurs through conserved amino acid motifs that include proximal histidine residues coordinating and stabilizing substrate positioning for catalysis [14]. Minor amino acid substitutions may influence both heme affinity and turnover rates [15].

Sequences available in databases such as NCBI provide opportunities for computational analysis. Tools such as Clustal Omega for multiple sequence alignment and MEGA for phylogenetic analysis are widely used to identify conserved residues and infer evolutionary relationships [16-17].

Previous studies have often focused on a limited number of strains or restricted geographic regions, and systematic comparisons across geographically diverse isolates remain limited. Similarly, integration of sequence analysis with phylogenetic assessment to understand functional implications is still lacking.

The current study aims to address these gaps by analyzing variations in the heme oxygenase protein sequences among *Leptospira interrogans* isolates from geographically diverse regions spanning 15 countries. Additionally, an in-depth analysis of sequence variation, evolutionary relationships, and potential structural divergence using bioinformatics tools was carried out.

Material and Methods

A total of 35 heme oxygenase protein sequences of *Leptospira interrogans* were retrieved from the NCBI GenBank database (http://www.ncbi.nim.nih.gov). The sequences were screened based on following criteria: (i) complete protein sequences, (ii) a clear geographical origin, and (iii) no ambiguous residues. The full-length protein sequences of 225 amino acids were selected and partial, truncated and duplicate sequences were eliminated from alignment. The dataset included isolates from 15 geographically diverse countries, namely USA, Panama, Colombia, Brazil, Argentina, China, Canada, France, Sri Lanka, Kazakhstan, India, Malaysia, Germany, Poland, and Thailand (Table 1). Multiple sequence

alignment was performed by using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalol) [18]. The alignment was manually scrutinised for proper alignment quality and conservation of key regions.

The phylogenetic analysis was performed by using MEGAX [19]. The evolutionary relationships were inferred using the Neighbor-Joining (NJ) method with the amino acid substitution model based on the number of differences [20]. The rates among sites were assumed to be uniform, and the pattern among lineages was considered homogeneous. The positions which contained gaps and missing data was treated as complete deletion. The tree was drawn to scale, with branch lengths depicting number of amino acid difference per sequence. The bootstrap value expressed as percentages of 1000 replications were calculated [21].

Table 1. List of *Leptospira interrogans* heme oxygenase protein sequences retrieved from the NCBI database, representing 35 strains across 15 geographically diverse countries along with their corresponding accession numbers.

Serial No	Country Name	Accession No.
1.	USA	XMY37433.1, ENO71500.1, XMT33852.1, SIP93380.1, XSA80053.1, WPM74573.1, KAA1293732.1
2.	Panama	QHH72948.1, QIP65945.1, QHH55600.1
3.	Colombia	OQN92017.1
4.	Brazil	KPA34531.1, ASV07998.1, ALE41833.1, YBV15930.1, UNS61305.1
5.	Argentina	POR20005.1, KYZ63858.1
6.	China	XMB56138.1, OQM33926.1
7.	Canada	TQE60646.1
8.	France	SOR63412.1, KWV22434.1
9.	Sri Lanka	UMQ56579.1
10.	Kazakhstan	XZE68316.1, WML95894.1
11.	India	KGE21280.1, EKO86886.1
12.	Malaysia	OAM81087.1
13.	Germany	QOI52809.1, QOI44755.1
14.	Poland	UPO19462.1, UQX09065.1
15.	Thailand	KAK2617346.1, QYY62499.1

Results

The heme oxygenase protein sequences of 35 strains from 15 countries were retrieved from NCBI database. On analysing the CLUSTAL Omega alignment several notable amino acid substitutions were found (Figure 1). A substitution of Phe84Ile was observed in the Brazilian strain (ASV07998.1). The substitution of Asp102Asn was found in a strain from France (SOR63412.1). The Lys105Gln substitution appeared in 12 isolates from six diverse countries particularly five from the USA (XMY37433.1, ENO71500.1, KAA1293732.1, XMT33852.1, XSA80053.1), one each from China (XMB56138.1), France (KWV22434.1), Thailand (QYY62499.1), India (EKO86886.1), and Malaysia (OAM81087.1). The Ala141Val substitution was reported in an isolate from Germany (QOI52809.1). The Pro178Leu substitution was observed in an isolate from USA (XSA80053.1). The Asp181Asn substitution was found in an isolate from Thailand (KAK2617346.1). Additionally, the Ile201Asn substitution was observed in an isolate from Brazil (ASV07998.1). Finally, the Val221Ile mutation was detected in two isolates from Kazakhstan (WML95894.1, XZE68316.1).

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ASV07998        NLVLKSIYFPELYRKNALLEDLQIFYGTWKPNDHQPSVATQDYVKRIRKISETQPELLAA    120
KAK2617346     NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVQRIRKISETQPELLAA    120
QOI52809        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVKRIRKISETQPELLAA    120
XZE68316        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVKRIRKISETQPELLAA    120
WML95894        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVKRIRKISETQPELLAA    120
SOR63412        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQNYVKRIRKISETQPELLAA    120
XSA80053        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVQRIRKISETQPELLAA    120
SIP93380        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVKRIRKISETQPELLAA    120
WPM74573        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVKRIRKISETQPELLAA    120
QHH72948        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVKRIRKISETQPELLAA    120
QIP65945        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVKRIRKISETQPELLAA    120
QHH55600        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVKRIRKISETQPELLAA    120
OQN92017        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVKRIRKISETQPELLAA    120
KPA34531        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVKRIRKISETQPELLAA    120
ALE41833        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVKRIRKISETQPELLAA    120
YBV15930        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVKRIRKISETQPELLAA    120
UNS61305        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVKRIRKISETQPELLAA    120
POR20005        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVKRIRKISETQPELLAA    120
KYZ63858        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVKRIRKISETQPELLAA    120
OQM33926        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVKRIRKISETQPELLAA    120
TQE60646        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVKRIRKISETQPELLAA    120
UMQ56579        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVKRIRKISETQPELLAA    120
KGE21280        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVKRIRKISETQPELLAA    120
QOI44755        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVKRIRKISETQPELLAA    120
UPO19462        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVKRIRKISETQPELLAA    120
UQX09065        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVKRIRKISETQPELLAA    120
XMY37433        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVQRIRKISETQPELLAA    120
KAA1293732      NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVQRIRKISETQPELLAA    120
XMT33852        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVQRIRKISETQPELLAA    120
XMB56138        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVQRIRKISETQPELLAA    120
KWV22434        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVQRIRKISETQPELLAA    120
EKO86886        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVQRIRKISETQPELLAA    120
OAM81087        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVQRIRKISETQPELLAA    120
QYY62499        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQDYVQRIRKISETQPELLAA    120
ENO71500        NLVLKSIYFPELYRKNALLEDLQFFYGTWKPNDHQPSVATQNYVQRIRKISETQPELLAA    120
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ASV07998        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
KAK2617346     HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
QOI52809        HSYVRYLGDLSGGQILKKVAVRALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
XZE68316        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
WML95894        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
SOR63412        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
XSA80053        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLLVN    180
SIP93380        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
WPM74573        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
QHH72948        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
QIP65945        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
QHH55600        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
OQN92017        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
KPA34531        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
ALE41833        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
YBV15930        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
UNS61305        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
POR20005        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
KYZ63858        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
OQM33926        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
TQE60646        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
UMQ56579        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
KGE21280        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
QOI44755        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
UPO19462        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
UQX09065        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
XMY37433        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
KAA1293732      HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
XMT33852        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
XMB56138        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
KWV22434        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
EKO86886        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
OAM81087        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
QYY62499        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
ENO71500        HSYVRYLGDLSGGQILKKVAARALNLPEGKGISFYEFPMIQDINGFKQNYRTALDSLPVN    180
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Figure 1. Multiple sequence alignment of 35 heme oxygenase protein sequences from *Leptospira interrogans* using CLUSTAL Omega (https://www.ebi.ac.uk/Tools/msa/clustalol). The "*" means the identical and fully conserved amino acid residue. ":" means conservation within strong group of amino acid residue. "." means conservation within weaker group of amino acid residues.

The Neighbor-joining tree constructed from heme oxygenase protein sequences of *Leptospira interrogans* from 15 different countries clustered with moderate bootstrap values from 40- 64% (Figure 2). The phylogenetic tree revealed that the isolates clustered into two major Clades - Clade I and Clade II. The Clade I comprised of isolates from Kazakhstan (WML95894.1 and XZE68316.1) in subclade IA followed by intermixed global isolates (WPM74573.1, YBV15930.1, UQX09065.1, UPO19462.1, UNS61305.1, UMQ56579.1, TQE60646.1, SIP93380.1) in subclade IB. The Subclade IC, a transitional cluster containing QOI52809.1, QOI44755.1, QIP65945.1, QHH72948.1, QHH55600.1, POR20005.1, OQN92017.1, OQM33926.1, KYZ63858.1, KPA34531.1, and KGE21280.1. was observed. The second major Clade II was distributed within four subclades- Subclade IIA comprised of ENO71500.1 and SOR63412.1 whereas Subclade IIB included EKO86886.1, KAA1293732.1, and KAK2617346.1. The third Subclade IIC contained the sequences from KWV22434.1, OAM81087.1, QYY62499.1, XMB56138.1, XMT33852.1, XMY37433.1, XSA80053.1 whereas a distinct terminal group formed Subclade IID consisted of ALE41833.1 and ASV07998.1

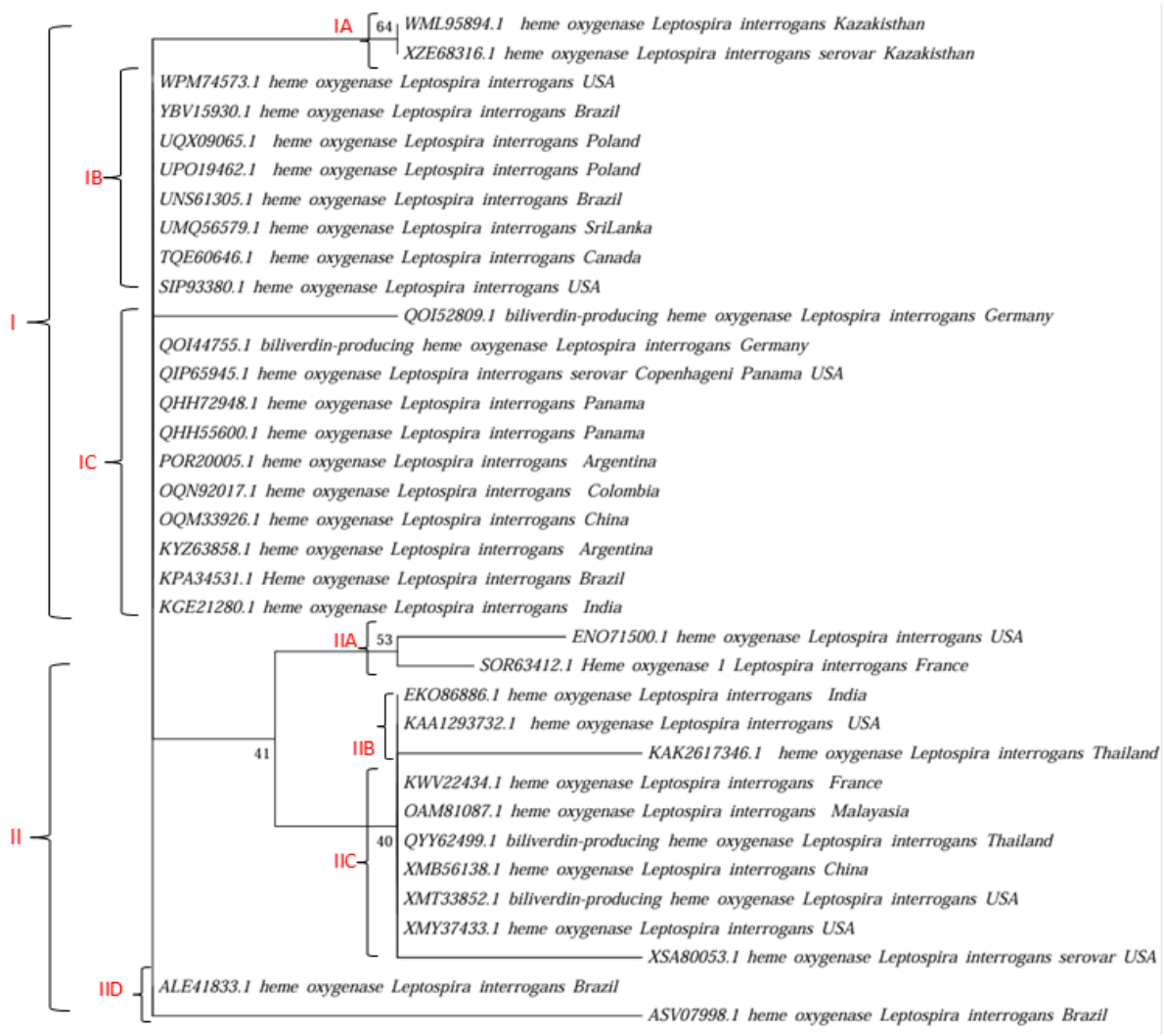

Figure 2. Phylogenetic tree generated by neighbor-joining method on the basis of full-length heme oxygenase protein sequences of the 35 strains of *Leptospira interrogans* sequences available in the database. The optimal tree with the sum of branch length is 8.50000000. Bootstrap value expressed as percentages of 1000 replications as shown at nodes. The clades and subclades have been indicated in red.

Discussion

The enzymatic catalysis of heme requires the binding of the bacterial heme oxygenase in a proper positioned manner [22-23]. The heme binding pocket in the enzyme is formed by residues within both distal helix and adjacent flexible loops [22, 24]. Mutations observed in this study that substitutions at various positions may have a moderate to drastic effect on the binding efficiency of enzyme and substrate [22-23]. The *in-silico* analysis of the protein sequences by alignment of amino acids revealed few key substitutions. A non-conservative substitution was observed at Phe84Ile in single protein sequence from Brazil [25]. The replacement of an aromatic amino acid with aliphatic one could maybe interfere with heme interaction and hence cause slightly reduced enzyme substrate binding affinity. However, in the absence of structural or binding analysis, its effect on substrate affinity remains hypothetical [26]. An amino acid substitution from aspartate to asparagine that was detected at 102nd and 181st positions in sequences suggests a shift in the loss of the negative charge to neutral one [22]. This might lead to reduced binding affinity to the substrate. Although

it suggests changes in electrostatic properties theoretically but strong structural and functional validation is required. Similarly, loss of a positive charge to a neutral amino acid was observed at the Lys105Gln in 12 isolates [23]. Such type of point mutations involving conversion of charged to neutral amino acids potentially disrupt the electrostatic interactions crucial in maintaining the active site architecture but its role has to be experimentally established [22,26].
Other substitution like Pro178Leu in one sequence could impact the structural stability of the enzyme. The role of proline is attributed to stabilization of helix turns and loops [22]. Furthermore, substitution from Ile201Asn could disrupt hydrophobic core and could affect the protein stability which can only be confirmed by further stability analysis research [27]. Few substitutions such as Ala141Val and Val221Ile were conservative and could have minimal effect on the stability and binding efficiency of the enzyme [25].

The repeated occurrence of the Lys105Gln substitution among geographically diverse isolates may signify a locus of variation; however, ascribing this to evolutionary pressures such as adaptation or pathogenicity necessitates further population-level and functional investigations [22]. Substitutions like Asp102Asn and Asp181Asn might also have an effect on local interactions, like hydrogen bonding networks, but evidence for functional implications need to be established [26].

In contrast, substitutions at positions 141, 178, 201, and 221 are distal from the active site and are unlikely to directly participate in heme binding [22,24]. However previous research has revealed a mutational analysis of residue at Phe157 located on distal site proved to be critical in optimum chemical and dynamical environment for the heme oxygenase reaction, being responsible for a long-range communication from enzyme's outer fringes to the active site [22,28]. In general, the possible functional effects of the amino acid changes that were seen should be treated as hypotheses that need to be tested more through structural modelling and experimental research.

The phylogenetic assessment indicated that heme oxygenase protein sequences of *Leptospira interrogans* from 15 geographically diverse isolates were overall conserved though small substitutions contributed to divergence into subclades [26, 29-30]. In this study two distinct clades were observed where Clade I showed an intermingling of Kazakhstan isolates in Subclade IA, global isolates in Subclade IB and Europe-Panama mixed cluster in Subclade IC. This type of results with shorter branch lengths suggests common ancestry along with less influence due to geographical isolation [31-34]. However, the basal subclade IA comprising of Kazakhstan isolates indicated early divergence within the entire sequence dataset.

On the other hand, Clade II showed comparatively more structured subclustering, with smaller groups matching to particular regional affiliations. The Clade IIA formed by the France and USA group whereas Clade IIB is associated with sequences mapped to India, USA and France. In addition, Clade IIC particularly clustered the Europe and Asia sequences. The Clade IID was shown by a terminal cluster with sequences from the Brazil. This pattern indicates moderate evolutionary divergences, which could be due to accumulated mutations over time [30,34-35].

In this study, moderate to low bootstrap values (40–64%) observed in the phylogenetic tree indicate limited statistical support for some clades, suggesting low phylogenetic resolution. This is possibly due to the high conservation of the heme oxygenase proteins across geographically diverse isolates of *Leptospira interrogans*, resulting in a reduced number of informative amino acid substitutions. These findings highlight strong evolutionary constraints on heme oxygenase, reflecting its essential functional role more than significant divergence driven by geographic separation [36-37]. It could however not eliminate the possibility of point mutations if accumulated could lead to changes in the functionality of the protein over a period of time [24].

Conclusion

The present research highlights that the heme oxygenase protein sequences of *Leptospira interrogans* remains highly conserved across geographically diverse isolates with limited amino acid substitutions contributing to minor

phylogenetic divergence. However, several non-conservative and charge altering amino acid substitutions were identified and their potential impact on enzyme stability, substrate binding affinity and active site conformation can be hypothesized. The complete validation to this hypothesis can only be obtained after further structural modelling and experimental proof. The phylogenetic assessment reflected low bootstrap values, suggests strong evolutionary constraints because of the major functional role of heme oxygenase in virulence rather than geographic divergence. Overall, the findings necessities further structural and biochemical studies and implicate the role of such research in understanding similar type of virulence proteins for designing therapeutics.

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