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PAN-GENOMIC ANALYSIS OF HUMAN INFECTING SEROTYPES OF *LISTERIA MONOCYTOGENES*: IDENTIFICATION OF PUTATIVE DRUG TARGETS

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ABSTRACT: Pan-genome analysis can identify the core genome, which is the total number of genes present in all strains of a bacterial species. Further essential genes can be identified in this core set of genes which gives us a minimal number of genes required for the survival of the bacteria. This set of genes can be analyzed to develop new antimicrobial agents against pathogenic, multidrug-resistant bacteria such as *Listeria monocytogenes* which causes several primary and secondary infections in humans. Pan-genome analysis of 38 strains of *Listeria monocytogenes* was performed to estimate 2331 genes in core genome, 1056 genes in the dispensable genome, and 152 genes in the strain-specific genome. Essentiality analysis of 2331 core genome proteins predicted 965 essential core-genome families (ECGFs). Furthermore, the identification of host non-homologous proteins using BLASTP with the *Homo sapiens* proteome resulted in 485 non-homologous essential core-genome families (NH-ECGFs). These putative proteins can be analyzed to identify novel drug targets that could generate broad-spectrum, safe and effective therapeutic agents against *Listeria monocytogenes* infections in humans.

INTRODUCTION: *Listeria monocytogenes* is a rod-shaped, Gram-positive, food-borne pathogenic bacterium that is responsible for listeriosis, CNS infection, sepsis, liver infection, meningitis, spleen infection, premature birth and abortions [1]. Several immune response-based diseases such as Inflammatory Bowel Disease (IBD) and Rheumatoid Arthritis (RA) have also been associated with *Listeria monocytogenes* infections [2, 3].

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Listeria monocytogenes infect humans through the intake of contaminated food and cause primary infection in the intestines. It invades the intestinal epithelium barrier and enters the bloodstream, from where it causes secondary infections at numerous sites such as the spleen, bones, liver, brain *etc.* [1] The regular treatment for Listeria infections was administration of antibiotics such as penicillin G and/or ampicillin along with aminoglycosides such as gentamicin or kanamycin.

Most *Listeria monocytogenes* strains were susceptible to antibiotics, but soon drug resistance was observed against tetracycline in 1988. Subsequently, many multidrug-resistant Listeria monocytogenes have been isolated from the environment, food samples and human gut/stool samples [4, 5].

Due to easier, quicker and cost-effective sequencing facilities, the sequenced microbial genome number has increased drastically over the last two decades. The pan-genome concept was defined by Tettelin and his co-workers as the complete set of genes present in any bacterial species. The pan-genome was further classified into core genome (genes present in all strains of a bacterial species), dispensable genome (genes found in two or more strains but not all strains of a bacterial species) and strain-specific genome (genes found exclusively in one strain) [6]. The pan-genome analysis has been employed previously in several bacterial species such as *Campylobacter jejuni* [7], *Escherichia coli* [8], Campylobacter [9], *Salmonella enterica* [10] and Vibrio [11]. This pan-genome analysis concept can be employed to identify novel drug targets in pathogenic bacteria, as performed in this study. Such studies have been previously performed in several bacterial species such as *Pseudomonas aeruginosa* [12], *Acinetobacter baumannii* [13], *Clostridium botulinum* [14], Helicobacter pylori [15], *Salmonella enterica* [16], *Streptococcus pneumonia* [17].

Here we present findings of comparative analysis of 38 *Listeria monocytogenes* strains known for causing infections in the human host and present their pan genome (including details of core genome, dispensable genome and strain specific genome). The core genome was analyzed for essentiality and non-homology with human host to identify non-homologous conserved essential genes of *Listeria monocytogenes*. This was used to predict putative drug targets that could be used to generate broad-spectrum, safe and effective therapeutic agents against *Listeria monocytogenes* infections in humans.

MATERIAL AND METHODS:

Genomes and Gene Annotations: A total number of 4513 genomes are available for *Listeria monocytogenes* at the National Centre for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov/genome/browse/), which consists of 289 complete genomes, 70 Chromosome, 2764 Contig, and 1390 Scaffold sequences. A total of 38 complete genome sequences (as listed in **Table 1**) were selected for this study, including *Listeria monocytogenes* strains from serotype 1/2a, serotype 1/2b and serotype 4b only, as these serotypes are known for causing infection in humans [18]. These strains cause infection in humans through non-vegetarian, dairy, and vegetarian sources [19]. The genomic features of these 38 genomes are presented in **Table 1.**

TABLE 1: GENOMIC FEATURES OF THE *LISTERIA MONOCYTOGENES* STRAINS INCLUDED IN THIS STUDY

S. no.	Organism Name	Organism Groups	Strain	Genes	Level	Assembly	Size (Mb)	GC%	CDS
1	*Listeria monocytogenes* serotype 1/2a str. 08-6569	Bacteria; Terrabacteria group; Bacillota	08-6569	3,088	Complete	GCA_000 513595.1	3.03	38	2,991
2	*Listeria monocytogenes* serotype 1/2a str. 08-6997	Bacteria; Terrabacteria group; Bacillota	08-6997	3,088	Complete	GCA_000 513635.1	3.03	38	2,991
3	*Listeria monocytogenes* serotype 1/2a str. 10-0815	Bacteria; Terrabacteria group; Bacillota	10-0815	3,088	Complete	GCA_000 513655.1	3.03	38	2,991
4	*Listeria monocytogenes* serotype 1/2a str. 08-6056	Bacteria; Terrabacteria group; Bacillota	08-6056	3,087		GCA_002 213585.1	3.03	38	2,990
5	*Listeria monocytogenes* serotype 1/2a str. 98-2035	Bacteria; Terrabacteria group; Bacillota	98-2035	3,084	Complete	GCA_002 213705.1	3.03	38	2,985
6	*Listeria monocytogenes* serotype 1/2a str. 99-6370	Bacteria; Terrabacteria group; Bacillota	99-6370	3,084		GCA_002 213725.1	3.03	38	2,985
7	*Listeria monocytogenes* serotype 1/2a str. 95-0093	Bacteria; Terrabacteria group; Bacillota	95-0093	3,041	Complete	GCA_002 213685.1	3	38	2,943
8	*Listeria monocytogenes* serotype 1/2a str. 04-5457	Bacteria; Terrabacteria group; Bacillota	04-5457	3,038		GCA_002 213565.1	3	38	2,939

9	*Listeria monocytogenes* serotype 1/2a str. 08-7374	Bacteria; Terrabacteria group; Bacillota	08-7374	3,038	Complete	GCA_002213605.1	3	38	2,939
10	*Listeria monocytogenes* serotype 1/2a str. 10-1321	Bacteria; Terrabacteria group; Bacillota	10-1321	3,039	Complete	GCA_002213665.1	3	38	2,941
11	*Listeria monocytogenes* serotype 1/2a str. 10-5024	Bacteria; Terrabacteria group; Bacillota	1141293	3,038	Complete	GCA_002213905.1	3	38	2,940
12	*Listeria monocytogenes* serotype 1/2a str. 10-0814	Bacteria; Terrabacteria group; Bacillota	10-0814	3,038	Complete	GCA_002214045.1	3	38	2,939
13	*Listeria monocytogenes* serotype 1/2a str. 02-5993	Bacteria; Terrabacteria group; Bacillota	02-5993	3,038	Complete	GCA_002213545.1	3	38	2,939
14	*Listeria monocytogenes* serotype 1/2a str. 10-1047	Bacteria; Terrabacteria group; Bacillota	10-1047	3,036	Complete	GCA_000513675.1	3	38	2,942
15	*Listeria monocytogenes* serotype 1/2a str. 10-1046	Bacteria; Terrabacteria group; Bacillota	10-1046	3,032	Complete	GCA_002213645.1	3	38	2,936
16	*Listeria monocytogenes* serotype 1/2a str. 88-0478	Bacteria; Terrabacteria group; Bacillota	88-0478	3,039	Complete	GCA_000513695.1	2.99	38	2,943
17	*Listeria monocytogenes* serotype 1/2a str. 08-7669	Bacteria;Terrabacteria group; Bacillota	08-7669	2,973	Complete	GCA_002213625.1	2.95	38	2,877
18	*Listeria monocytogenes* serotype 1/2a str. 10-0812	Bacteria; Terrabacteria group; Bacillota	10-0812	2,967	Complete	GCA_002214125.1	2.94	38	2,879
19	*Listeria monocytogenes* serotype 1/2a str. 10-0813	Bacteria; Terrabacteria group; Bacillota	10-0813	2,967	Complete	GCA_002214145.1	2.94	38	2,878
20	*Listeria monocytogenes* serotype 1/2a str. 10-4754	Bacteria; Terrabacteria group; Bacillota	10-42677	2,927	Complete	GCA_002213805.1	2.91	38	2,833
21	*Listeria monocytogenes* serotype 1/2a str. 10-4758	Bacteria; Terrabacteria group; Bacillota	10-44138	2,926	Complete	GCA_002213825.1	2.91	38	2,832
22	*Listeria monocytogenes* serotype 1/2a str. 10-0933	Bacteria; Terrabacteria group; Bacillota	10-0933	2,866	Complete	GCA_002213845.1	2.87	38	2,770
23	*Listeria monocytogenes* serotype 1/2a str. 10-0934	Bacteria; Terrabacteria group; Bacillota	10-0934	2,867	Complete	GCA_002213865.1	2.87	38	2,773
24	*Listeria monocytogenes* serotype 1/2b str. 10-0810	Bacteria; Terrabacteria group; Bacillota	10-0810	3,037	Complete	GCA_002214085.1	3.02	38	2,940
25	*Listeria monocytogenes* serotype 1/2b str. 10-0811	Bacteria; Terrabacteria group; Bacillota	10-0811	3,037	Complete	GCA_002214105.1	3.02	38	2,940
26	*Listeria monocytogenes* serotype 4b str. 02-1103	Bacteria; Terrabacteriagroup; Bacillota	02-1103	2,982	Complete	GCA_002213885.1	2.98	37.9	2,880
27	*Listeria monocytogenes* serotype 4b str. F2365	Bacteria; Terrabacteriagroup; Bacillota	4b F2365	2,894	Complete	GCA_000008285.1	2.91	38	2775
28	*Listeria monocytogenes* serotype 4b str. 02-1103	Bacteria; Terrabacteriagroup; Bacillota	02-1103	2,982	Complete	GCA_002213885.1	2.98	37.9	2880
29	*Listeria monocytogenes* serotype 4b str. 02-1289	Bacteria; Terrabacteriagroup; Bacillota	02-1289	2,982	Complete	GCA_002213925.1	2.98	37.9	2880

30	*Listeria monocytogenes* serotype 4b str. 02-1792	Bacteria; Terrabacteriagroup; Bacillota	02-1792	2,982	Complete	GCA_002213945.1	2.98	37.9	2880
31	*Listeria monocytogenes* serotype 4b str. 81-0592	Bacteria; Terrabacteriagroup; Bacillota	81-0592	2,985	Complete	GCA_002214005.1	2.98	38	2882
32	*Listeria monocytogenes* serotype 4b str. 10-0809	Bacteria; Terrabacteriagroup; Bacillota	10-0809	2,982	Complete	GCA_002214065.1	2.98	38	2879
33	*Listeria monocytogenes* serotype 4b str. 81-0861	Bacteria;Terrabacteriagroup;Bacillota	81-0861	2,983	Complete	GCA_000513615.1	2.97	38	2868
34	*Listeria monocytogenes* serotype 4b str. 81-0558	Bacteria; Terrabacteriagroup; Bacillota	81-0558	2,979	Complete	GCA_002213985.1	2.97	38	2876
35	*Listeria monocytogenes* serotype 4b str. CLIP 80459	Bacteria; Terrabacteriagroup; Bacillota	Clip80459	2,889	Complete	GCA_000026705.1	2.91	38.1	2783
36	*Listeria monocytogenes* serotype 4b str. 02-6679	Bacteria; Terrabacteriagroup; Bacillota	02-6679	2,883	Complete	GCA_002213505.1	2.91	38	2788
37	*Listeria monocytogenes* serotype 4b str. 02-6680	Bacteria; Terrabacteriagroup; Bacillota	02-6680	2,883	Complete	GCA_002213965.1	2.91	38	2788
38	*Listeria monocytogenes* serotype 4b str. LL195	Bacteria; Terrabacteriagroup; Bacillota	LL195	2,890	Complete	GCA_000318055.1	2.90	38	2776

This information has been collected from the NCBI database (www.ncbi.nlm.nih.gov/genome/browse/). CDS: Protein Coding Sequence.

Pan-genomic Analysis: CMG-biotools program was used to determine the pan-genome of 38 *Listeria monocytogenes* [10, 20]. Proteomes were constructed for all 38 genomes and pair-wise proteome comparison was performed using BLASTP algorithm (Protein-protein Basic Local Alignment Search Tool) to see if two proteins are shared among genomes [21, 22]. Any two proteins were considered to be conserved or be in the same family if they follow the "50/50 rule" that says 50% of the alignment contain identical matches and the length of the alignment is 50% of the longest gene (50% identity/50% gene length). A number of new genes was recorded at the sequential addition of every new genome. Each new gene was compared to the existing genome representative using the 50/50 rule. If the new gene satisfies the 50/50 rule, it becomes a core or pan gene family. Genes not fulfilling this criterion were assigned a unique family (singletons) [10, 20]. The terms gene(s) and protein(s) are used interchangeably in this paper.

Identification of Essential Core-genome Families (ECGFs): The protein sequences from the core genome families (obtained from the pan-genome analysis) were further subjected to BLASTP analysis against the database of essential genes (DEG; http://www.essentialgene.org/) [23]. The criteria of essentiality are as follows: evalue< 1e-10, bit score ≥ 100 and percentage identity ≥ 35 [24, 25]. The protein sequences that satisfied the essentiality criteria were considered essential core genome families (ECGFs). The essential gene set is important to determine the cellular processes crucial to the organism's existence. Identifying ECGFs is important for developing broad-spectrum drug targets, as targeting these proteins will produce lethal phenotypes.

Identification of Non-homologous Essential Core-genome Families (NH-ECGFs): The protein sequences of ECGFs were subjected to BLASTP analysis with the *Homo sapiens* proteome. The protein sequences with an e-value cut-off of < 1e-4 were considered homologous to the pathogen and excluded from the study. The protein sequences without a hit under this criterion were considered to have no significant homolog in *Homo sapiens* selected for further analysis as non-homologous essential core-genome families (NH-ECGFs) [24].

Subcellular Localization of NH-ECGFs Proteins: PSORTb version 3.0

(http://www.psort.org/psortb) was used to predict the precise bacterial protein subcellular localization (SCL) for the NH-ECGFs proteins obtained at the previous step. It predicted four localizations: cytoplasmic, cytoplasmic membrane, cell wall and extracellular for our Gram-positive pathogenic bacteria [26]. The research design for this study has been presented in **Fig. 1**.

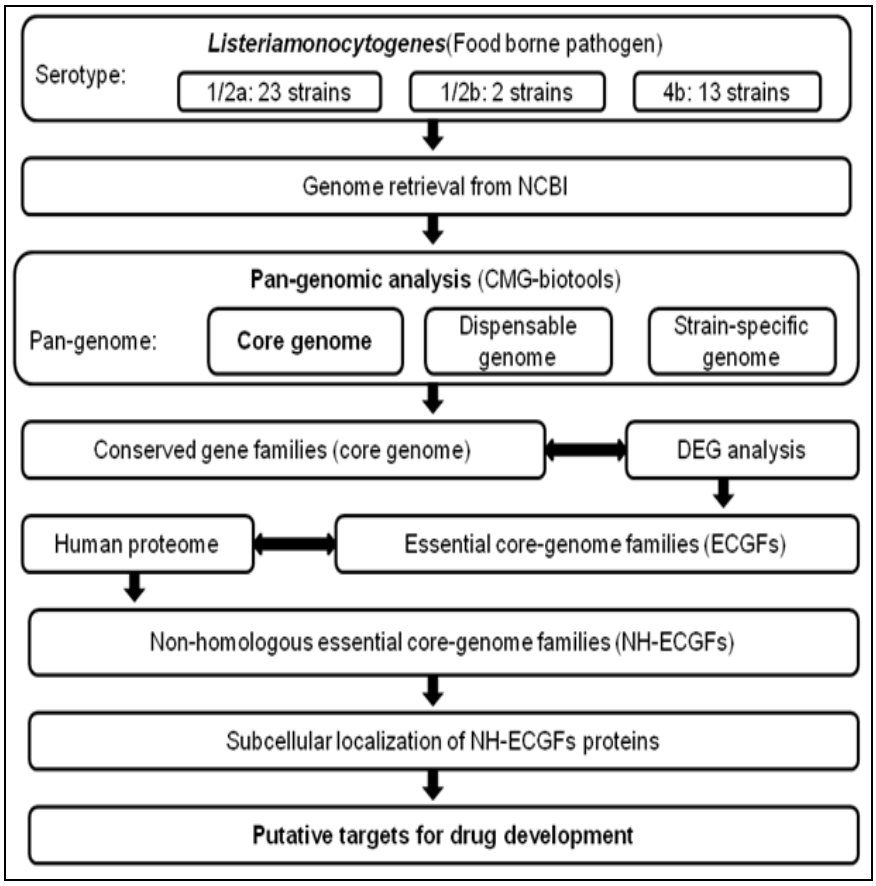

FIG. 1: RESEARCH DESIGN

RESULTS AND DISCUSSION:

Genomes and Gene Annotations: 4517 genomes for species *Listeria monocytogenes* are available at NCBI and *Listeria monocytogenes* EGD-e is the reference genome in this study. The *Listeria monocytogenes* genome has a median total length of 2.97225 (Mb); median protein count of 2896 and a median GC% of 37.9. The genomic features for the selected 38 strains are compiled in **Table 1**. These 38 strains are the complete genomes available at NCBI, belonging to the serotype 1/2a, serotype 1/2b and serotype 4b. These serotypes are known to cause human infections and were therefore selected for this study.

Pan-genomic Analysis: The pan-genome analysis for our 38 *Listeria monocytogenes* strains performed by CMG-biotools revealed the pan genome size of 3539 genes (*i.e*. the total number of gene families present for the selected genomes). The pan-genome was further classified into core genome (genes present in all strains of a bacterial species), dispensable genome (genes found in two or more strains but not all strains of a bacterial species), and strain-specific genome (genes found exclusively in one strain) [6]. These 3539 gene families were further bifurcated to 2331 genes in the core genome (66% of the pan-genome); 1056 genes in the dispensable genome (30% of the pan-genome); and 152 genes in the strain-specific genome (4% of the pan-genome) (as presented in **Table 2**). These gene families have been further classified into annotated proteins (APs) and hypothetical Proteins (HPs). APs are the proteins whose structural and functional information has been deduced along with sequencing through several analysis, comparison, and mining techniques. Whereas, HPs are the proteins that are predicted to be expressed but no experimental evidence of their translation or existence has been presented [27]. HPs are important, as they fall into

functional gene families and might be linked with several human diseases. Regardless of the lack of their functional characterization, they might play a significant role in understanding physiological and biochemical pathways [28, 29]. The core genome family in our analysis consisted of 1553 APs (67%) and 778 HPs (33%). The dispensable and strain-specific genome analysis gave 862 APs; 194 HPs and 138 APs; 14 HPs, respectively **Table 2**.

TABLE 2: DETAILS OF PAN-GENOME FOR SELECTED 38 STRAINS OF *LISTERIA MONOCYTOGENES*

Genomes of 38 strains of *Listeria monocytogenes (Pan genome size 3539 genes)*					
Core genome 2331 gene families (66%)		**Dispensable genome 1056 gene families (30%)**		**Strain-specific genome 152 gene families (4%)**	
Annotated proteins (APs)	**Hypothetical Proteins (HPs)**	**Annotated proteins (APs)**	**Hypothetical Proteins (HPs)**	**Annotated proteins (APs)**	**Hypothetical Proteins (HPs)**
1553 (67%)	778 (33%)	862 (82%)	194 (18%)	138 (91%)	14 (9%)

The core genome was selected for further analysis to find putative drug targets. These gene families are present in all the strains of pathogenic *Listeria monocytogenes* and could be targeted to develop broad-spectrum drugs active against all these infectious strains. The list of all the 2331 gene families (and their details) of the core genome has been provided in Supplementary **Table 1**.

Essentiality Analysis (ECGFs): Essential genes are the minimal number of genes obligatory for any organism's survival [30]. Essential genes have been determined for 48 bacterial species and have been listed in the Database of Essential Genes (DEG). The essential genes list can be directly extracted for these organisms and BLAST analysis can be performed. Alternatively, a common list of prokaryotic essential genes can be used to analyze other organisms not listed in DEG. (DEG; http://www.essentialgene.org/). This essentiality test was performed on 2331 proteins of the core genome and 965 proteins were found to be essential, referred to as essential core-genome families (ECGFs) **Table 3**. Out of these 965 ECGFs, 853 proteins were APs and 112 proteins were found to be HPs (presented in **Table 3**). The essential gene set is important to determine the cellular processes critical to the organism's existence.

The knockout of any essential bacterial gene can produce lethal phenotypes, so the essential genes may act as significant drug targets [23, 31]. This can also be exploited to generate specific drug targets or vaccines against multidrug-resistant strains such as *Listeria monocytogenes*. Some essential genes may be conserved over several related species and are potential targets for the development of broad-spectrum antibiotics [23, 32]. The complete list and details of the 965 ECGFs have been presented in Supplementary **Table 2**.

TABLE 3: DETAILS OF ESSENTIAL CORE-GENOME FAMILIES (ECGFS) AND NON-HOMOLOGOUS ESSENTIAL CORE-GENOME FAMILIES (NH-ECGFS) OBTAINED BY PERFORMING BLASTP OF CORE GENOME WITH DEG AND HUMAN PROTEOME RESPECTIVELY

Core genome: 2331 gene families			
Essential core-genome families (ECGFs) 965		**Non-homologous essential core-genome families (NH-ECGFs) 485**	
No. of Hypothetical Proteins (HPs)	**No. of Annotated proteins (APs)**	**No. of Hypothetical Proteins (HPs)**	**No. of Annotated proteins (APs)**
112 (12%)	853 (88%)	21 (4%)	464 (96%)

Analysis of Human Non-homologous (NH-ECGFs): The 965 ECGFs obtained from the previous step were then compared (BLASTP) with the *Homo sapiens* proteome to identify host non-homologous proteins, which can be the potential drug targets. Proteins that do not show any significant homology to the host *Homo sapiens* proteome may act as effective drug targets as these drug/vaccine candidates have reduced risk of any unnecessary interaction with the host proteins. Hence, these drugs will be harmless and not adversely affect the human host metabolism. Out of the 965 ECGFs, 485 were found non-homologous to the *Homo sapiens* proteome and were referred as

Non-homologous essential core-genome families (NH-ECGFs). Further, 464 NH-ECGFs were annotated and rest 21 were hypothetical proteins, as compiled in **Table 3**. The list of 485 NH-ECGFs has been provided in Supplementary **Table 3**.

Subcellular Localization Analysis: Subcellular localization of the 485 NH-ECGFs was determined using PSORTb version 3.0. Out of 485 NH-ECGFs, PSORTb predicted 352 as cytoplasmic protein, 109 ascytoplasmic membrane protein; 1 as extracellular protein, whereas 23 proteins remained with unknown localization. No proteins were found to be localized in the bacteria's cell wall (as depicted in **Fig. 2**). The complete detail of the subcellular localization of 485 NH-ECGFs has been compiled in Supplementary **Table 4**. Subcellular localization of the putative drug target is important as it may provide important information about the function of these proteins. Cytoplasmic proteins are more favourable drug targets as they have plenty of enzymes, whereas membrane-bound and extracellular proteins are suitable as vaccine targets [33].

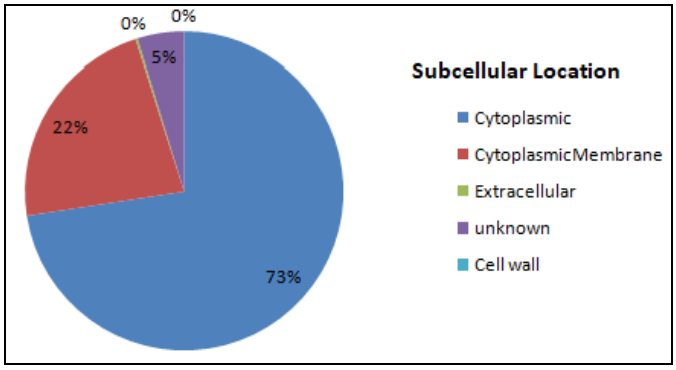

FIG. 2: SUBCELLULAR LOCALIZATION OF THE PREDICTED NH-ECGFS PROTEINS USING PSORTB VERSION 3.0.

CONCLUSION: In this study, we identified putative drug targets for serotypes of *Listeria monocytogenes* which are known to cause infections in humans. Core genome proteins were targeted for this, which was further subjected to essentiality analysis to find broad-spectrum drug targets. The downstream analysis included identifying host non-homologous proteins and subcellular localization analysis to discover safe and effective drug targets. Here we present 485 Non-homologous essential core-genome proteins (out of which 352 proteins have cytoplasmic localization) as putative drug targets in human infecting serotypes of *Listeria monocytogenes*. These proteins can further be analyzed and refined through a network biology approach to see their interaction with each other and host proteins for identifying novel drug targets against *Listeria monocytogenes.*

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CONFLICTS OF INTEREST: The authors declare no conflict of interest.

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