Comparative Immunology, Microbiology and Infectious Diseases 86 (2022) 101815

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Global epidemiology of CTX-M-type β-lactam resistance in human and animal

Chanchal Rana [a], Shiveeli Rajput [a], Manisha Behera [a,b], Devika Gautam [a], Vaibhav Vikas [c], Ashutosh Vats [a], Mayank Roshan [a], Soma M. Ghorai [b], Sachinandan De [a,*]

[a] ICAR - National Dairy Research Institute (NDRI), Animal Biotechnology Centre, Animal Genomics Lab, Karnal 132001, Haryana, India
[b] Department of Zoology, Hindu College, University of Delhi, Delhi 110007, India
[c] National Institute of Technology Tiruchirappalli (NIT Trichy), Tamil Nadu 620015, India

ARTICLE INFO

Keywords:
β-lactam
β-lactamase
CTX-M
ESBL
AMR
Antibiotic resistance

ABSTRACT

CTX-M ESBL are widely found in animal and human infections. For better understanding of CTX-M variations and epidemiology, a total of 2210 CTX-M sequences were retrieved from NCBI as at 20 December 2020. The maximum incidences of CTX-M were reported in China ($n = 508$), USA ($n = 354$) and Japan ($n = 180$). Single amino acid substitution in the domain region of CTX-M ESBL lead to survival benefits to the bacteria. A total of 31 different variations were found of which D240G was the most common followed by A77V and V103I substitutions. The variations in CTX-M enzymes were explained continent-wise revealing the maximum variation reported in America followed by Asia and Europe of which D240G substitution was the most prevalent. India contained only three variations (E166A, P167S D240G) found in New Delhi, Karnataka, West Bengal and Tamil Nadu. The P167 and D240 were under strong positive selection with dN/dS calculation.

1. Introduction

The β-lactams are the most commonly used class of antibiotics for the treatment of human and animal infections due to their broad activity, lack of toxicity and high level of tolerance [1,2]. They interrupt bacterial cell wall formation by targeting Penicillin-binding proteins (PBPs), inhibiting the creation of peptidoglycan crosslink and result in bacterial cell lysis [3]. The penicillin was the first β-lactam to be introduced in the year 1929 [4]. The penicillin antibiotics are used to treat bacterial illnesses such as pneumonia, respiratory tract infections and throat infections [5]. The penicillin, cephalosporins, carbapenems and monobactams are the four primary forms of β-lactams now available, three of which have bicyclic structures [4].

The resistance has spread as result of the increased use of the β-lactams, which can be due to mutation or horizontal gene transfer (HGT) [6]. Vast variants of bacterial species undergoing HGT and having short doubling time has led to the accumulation of a number of resistance type [7]. The development of β-lactamase enzymes in Gram-positive and Gram-negative bacteria causes resistance to β-lactam antibiotics, with latter being the most common source [4].

The Bush et al. introduced the Bush-Jacoby-Medeiros system in 1995 to classify them based on their functional properties [8]. Another classification was proposed by Ambler in 1980, which divided β-lactamase into four classes based on sequence information: Class A, B, C, and D [9]. The classes A, C, and D are serine β-lactamase (SBLs) which hydrolyze β-lactams by utilizing serine for nucleophilic attack at the amide carbonyl carbon via formation of covalent acylenzyme intermediate [4]. The class B zinc metalloenzymes (MBLs) are subdivided into three types: subclass B1, B2 and B3, which use water as a nucleophile activated by metal for β-lactam hydrolysis [4].

The Class A β-lactamase are mainly found on episomes which can transmit via conjugation leading to its wide dissemination [10]. The Extended-spectrum β-lactamases (ESBLs), generated via acquisition of point mutation, hydrolyze broad-spectrum cephalosporins and monobactams [11]. The spread of ESBLs including SHV, TEM, CTX-M severely limit the effectiveness of cephalosporins and cause a great threat to public health [4,11]. The CTX-M ESBLs are active against first, second and third generation cephalosporins.

According to Ambler classification, CTX-M enzymes are classified into five major groups: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 based on amino acid sequence similarity with $< 90\%$ amino acid identity between clusters and $> 95\%$ identity within clusters [12]. The

---

* Correspondence to: AGL, Animal Biotechnology Center, ICAR-National Dairy Research Institute, Karnal, HR, India.
E-mail address: sachinandan@gmail.com (S. De).

https://doi.org/10.1016/j.cimid.2022.101815
Received 18 January 2022; Received in revised form 5 April 2022; Accepted 12 April 2022

CTX-M enzymes are likely to have originated from *Kluyvera* species disseminated via mobile genetic elements including transposons, integrons, and IS elements, particularly ISEcp1, have played a significant role in their global spread [13]. The spread of CTX-M was exponential, leading to a wide diversity of CTX-M strains, resulting in "CTX-M pandemic" [14]. The CTX-M enzymes are disseminated globally, with CTX-M-15 and CTX-M-14 being the most common enzymes of CTX-M-1 and CTX-M-9 groups respectively [15,16]. In India, the CTX-M-15 is the most prevalent variant. The very first report came from Batra hospital and Medical Research Centre, New Delhi [17].

There are two subdomains in CTX-M protein structure. One is the alpha subdomain; consists mainly of alpha-helices and the other is alpha-beta subdomain consists of both alpha helices and beta-strands [18]. The groove formed by the two subdomains forms the active site. Within the active site, there are certain amino acid motifs (70STSK73, 103VNYN106, 130SDN132, 234KTG236) which are important for the recognition of substrate and hydrolytic processes [18,19].

The prevalence of CTX-M ESBL variants has increased in healthcare settings differing between continents. The various amino acid variations present in the active sites of the CTX-M protein play an important role in the enhancement or modulation of the hydrolytic activity of these enzymes. The CTX-M enzymes have succeeded in mobilizing to a number ecological niche, selective force, being the major factor [20]. It is important to study the effect of selection pressure on ESBL producing bacteria by identifying the genetic variation in CTX-M enzyme. The present work is aimed to analyze (i) the variations in CTX-M amino acid residues (ii) distribution of CTX-M variations globally including Indian subcontinent (iii) selection pressure acting on any specific domain of CTX-M.

2. Methods

2.1. Data acquisition

All available nucleotide sequences encoding CTX-M from animal and human infections were retrieved from NCBI database (https://www.NCBI database.nlm.nih.gov/) as at 20 December 2020 by using BLASTn tool (earlier available at Lahey database) (http://www.lahey.org/Studies/other.asp) (Supplementary data S1). The list of nucleotide accession numbers was used as an input for the Python script which uses selenium package to search NCBI for the corresponding amino acid sequences (Supplementary data S2). A total of 2210 CTX-M sequences were retrieved including all the variants. There were total 242 CTX-M variants reported till the date of retrieval of data. The 242 sequences representing each variant of CTX-M was selected for further studies.

2.2. Sequence analysis

Sequence analyses was performed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) implemented in MEGA-X software by applying MUSCLE algorithm with gap opening penalty of 1.20 and a gap extension penalty of 0 for Multiple sequence alignments (MSA) [21]. Subsequently, Neighbor-joining phylogeny tree was constructed using poisson substitution model and the robustness of the relevant nodes were estimated using 1000 replications (Bootstrap value = 1000). The NCBI reference sequence of CTX-M, accession number WP_013188473.1 was used as the canonical sequence for the analysis of CTX-M protein variants. FigTree v1.4.4 (http://tree.bio.ed.ac.uk/) was used for better representation of phylogenetic tree.

2.3. Motif evaluation

By studying the literature, motifs were generated using all 2210 retrieved CTX-M amino acid sequences. Variability at each site of motifs were evaluated for all novel 242 amino acid sequences using MEGA-X software. The frequency of variation at each site was determined manually and the CTX-M type harboring the mutations were noted.

2.4. Geographical Variation Analysis

All 2210 sequences were classified country-wise and further the sequences were classified into five groups of CTX-M (CTX-M-1, −2, −8, −9, −25) (Supplementary data S3). The reductant sequences were pooled. Using Data Driven Documents (https://d3js.org/), world map was prepared showing the highest to lowest cases of CTX-M prevalence globally (Fig. 1). The country-wise data was clumped into continents. The sequences were analyzed for variation using Mega-X software.

2.5. Selection pressure evaluation

The HyPhy software package implemented in Datamonkey web server was used to analyze all 2210 nucleotide sequences encoding CTX-M. Different selection methods were performed including (i) FEL (Fixed-Effects likelihood), (ii) FUBAR (Fast Unconstrained Bayesian AppRoximation), (iii) SLAC (Single-Likelihood Ancestor Counting) and MEME (Mixed Effect Likelihood Model) to evaluate selection pressure acting on different motifs of CTX-M. To classify a site as positively or negatively selected, a threshold p-value was set at 0.1 for SLAC, FEL AND MEME. For FUBAR, the threshold value for posterior probability was set at 0.9. The value of $\omega = dN/dS > 1$ (non-synonymous/synonymous substitution) indicates positive selection of the amino acid residues and $\omega = dN/dS < 1$ indicates negative selection of the amino acid residue.

3. Results

3.1. Classification of CTX-M sequences

Earlier, only 134 CTX-M sequences were phylogenetically classified into five Ambler groups [22]. A total of 242 CTX-M variants has been enlisted till 20 December 2020 in NCBI, which has been classified in this study (Fig. 2). The phylogenetic tree reflected the relationship of the CTX-M sequences and revealed the respective clades (Fig. 2). The Group 1 showed the highest number of CTX-M variants which suggests that the CTX-M-1 has undergone maximum mutations acquired over the period. The group 1 clade includes 116 CTX-M members, the group 2 clade contains 27 CTX-M sequences, group 8 clade include only 4 CTX-M members, group 9 clade include 71 members and group 25 clade include 14 CTX-M sequences. The phylogenetic tree created using neighbor-joining method displayed CTX-M-132, − 123, − 153, − 234, − 64 and CTX-M-199 in a separate clade, next to group 1 clade. Among these CTX-M-64, CTX-M-132 and CTX-M-123 have been reported earlier as CTX-M-1/9 hybrids and CTX-M-199 is a variant of CTX-M-64 [23,24]. The tree represented CTX-M-137 in group 9 clade, although earlier studies suggest it as a CTX-M-1/9 hybrid [25]. Clearly, CTX-M-1 group is the most diversified group, followed by CTX-M-9, suggesting that CTX-M-1 group members are more prone to mutations.

3.2. Variation in CTX-M amino acid residues

A total of 2200 sequences were analyzed. These sequences were narrowed down to 242 unique allelic variants of CTX-M. The multiple sequence alignment revealed characteristic residues and motifs which are important for hydrolyzing the amide bond of be β-lactam ring. A total of 20 amino acid sites were detected containing 31 different types of mutations in the domain region (from Ambler position 70–235) of 291 amino acid long CTX-M (Fig. 3). The highest substitution was found at position 240 (D240G), which does not fall into any secondary structure or motif region. A total of 221 sequences were found with variations in the residues important for hydrolysis of β-lactam ring. Most of them possessed D240G mutation (101 of 242 sequences) followed by A77V variations (45 of 242 sequences).

A total of four motifs are present in the active site CTX-M protein

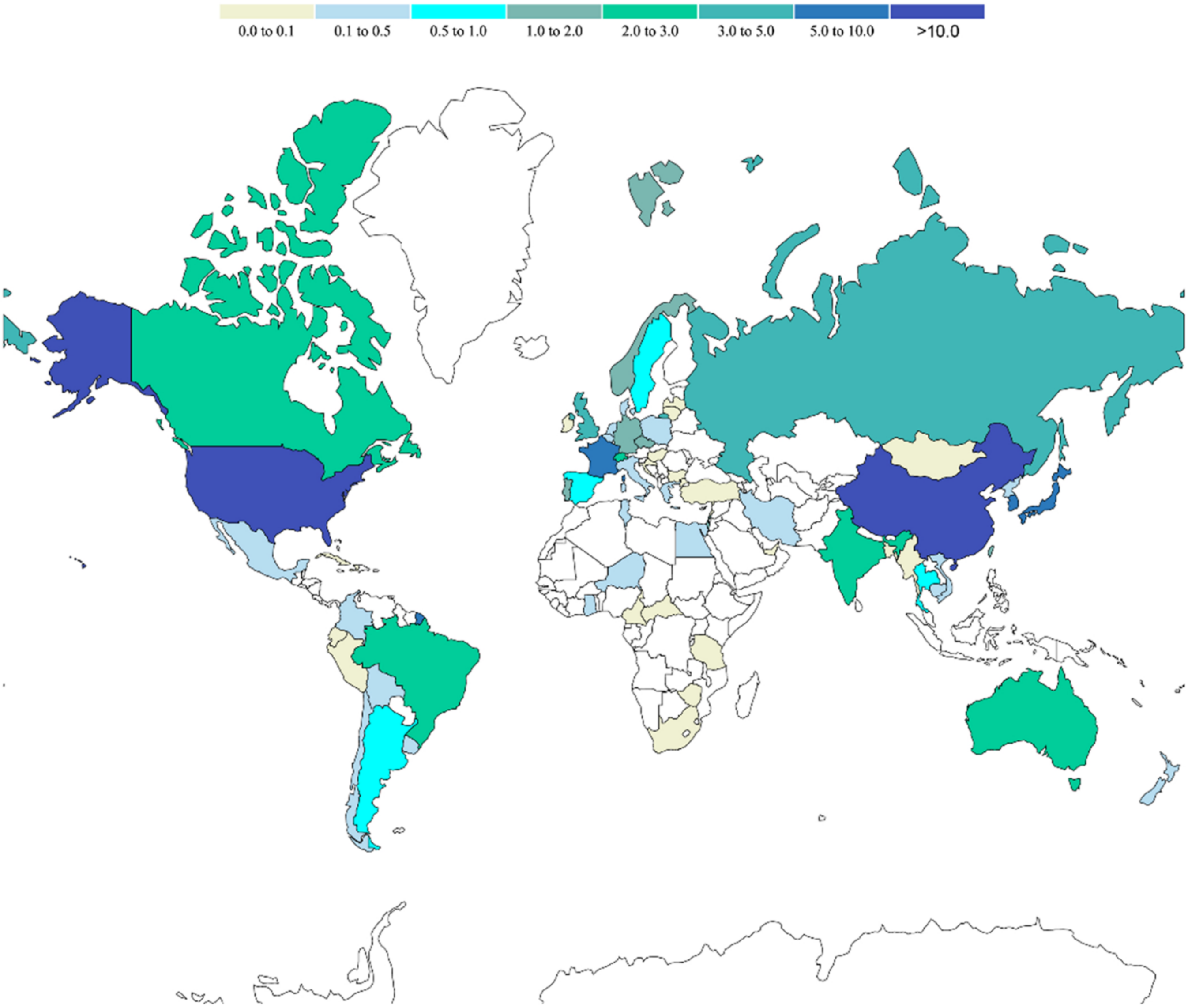

Fig. 1. Distribution of CTX-M worldwide (n = 2210). Scale represents highest to lowest number of CTX-M cases reported. Most of the sequences were obtained from China (n = 508), USA (n = 354) and Japan (n = 180) in a descending order of frequency.

structure namely 70STSK73, 103VNYN106, 130SDN132, 235KTG237 [9,18,26]. They play an important role in hydrolysis of β-lactam amide bond [27]. Therefore, this study further analyzed these four motifs for variations in amino acid residues. The 70STSK73 motif was found to be 100% conserved in all the CTX-M protein. In 103VNYN106 motif, N104 was found to be mutated to Tyr in CTX-M-201. The Y105 appeared to be 100% evolutionarily conserved amino acid. The N106 was found to be altered to Ser in CTX-M-33, CTX-M-67, CTX-M-195, CTX-M-217, CTX-M-220, CTX-M-223. In SDN motif (Ser130-Asp-Asn132), Asp131 appeared 100% conserved amino acid residue. The amino acid at position 130 shows two different types of variations, S130T mutation in CTX-M-190, CTX-M-199, CTX-M-218 and S130G mutation in CTX-M-190, CTX-M-199, CTX-M-218, CTX-M-234. The Asn132 was found to be substituted to Asp in CTX-M-127, 191, 215 and to His in CTX-M-181, CTX-M-81. In KTG motif, Lys234 was found to be mutated to Arg in CTX-M-203, CTX-M-106, CTX-M-192, CTX-M-107, CTX-M-146, CTX-M-239. The Gly236 was mutated to Ser in CTX-M-178, CTX-M-187, CTX-M-227. The Thr235 appeared 100% conserved in all CTX-M protein.

Within the Ω loop (Ambler positions 164–179), 31 out of the 242 CTX-M variants were found to harbor variations in the omega loop. There was a total of 18 type of variations in 31 variants since some substitutions were found to be present in more than one variant (Fig. 3). The Glu166 of Ω loop is involved in substrate binding and is strictly conserved residue but is mutated to Ala in CTX-M-155 and CTX-M-157. The effect of this variation is still not known. The Pro167 was found to be substituted to Ser in CTX-M-19, CTX-M-35, CTX-M-62, CTX-M-99, CTX-M-147, CTX-M-219, CTX-M-221.

There are other critical amino acids apart from the motif region which are important for the hydrolysis reaction, which includes Ala77, Val231, Asp240. The Val231 was found to be mutated to Ala in CTX-M-9 leading to decreased stability and catalytic activity of the protein towards CTX (Cefotaxime) but have improved the activity against ceftazidime [28,29]. The Asp240 residue was found to be mutated to Gly in members of all CTX-M groups.

Multiple sequence alignment of the available 2210 CTX-M sequences was performed and 31 substitutions were found in the domain region (from Ambler position 70–235) of CTX-M. Notably, most of the sequences were deposited from China (n = 508), USA (n = 354) and Japan (n = 180). The geographical prevalence patterns of CTX-M sequences containing different amino acid variations reveals that the maximum variants were reported from North America (23 of 31

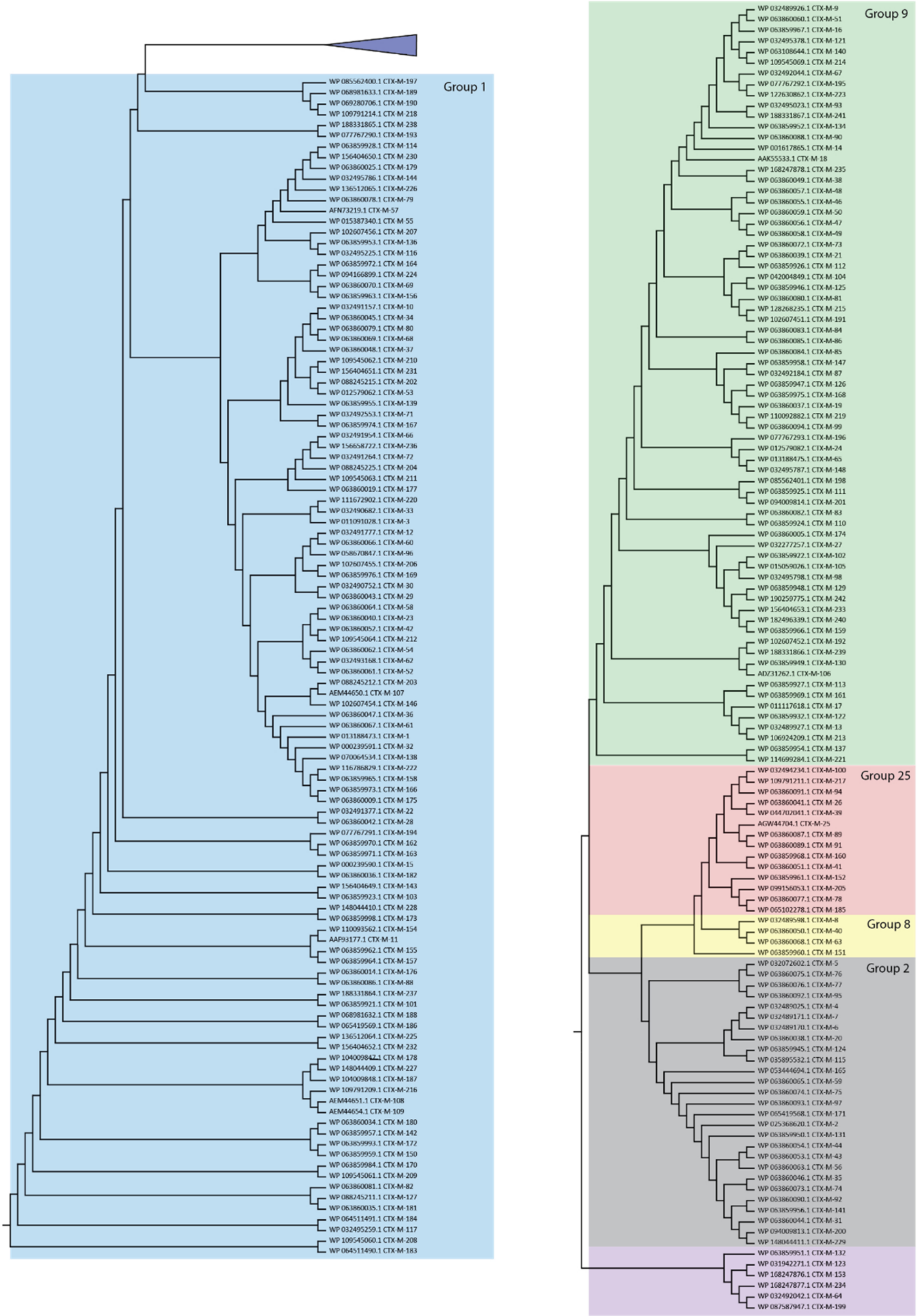

Fig. 2. The phylogenetic tree is constructed based on Neighbor-joining method with bootstrap value of 1000. A total of 242 CTX-M amino acid sequences is grouped into five clades. The order of groups is as followed: group 9, group 25, group 8, group 2 and group 1.

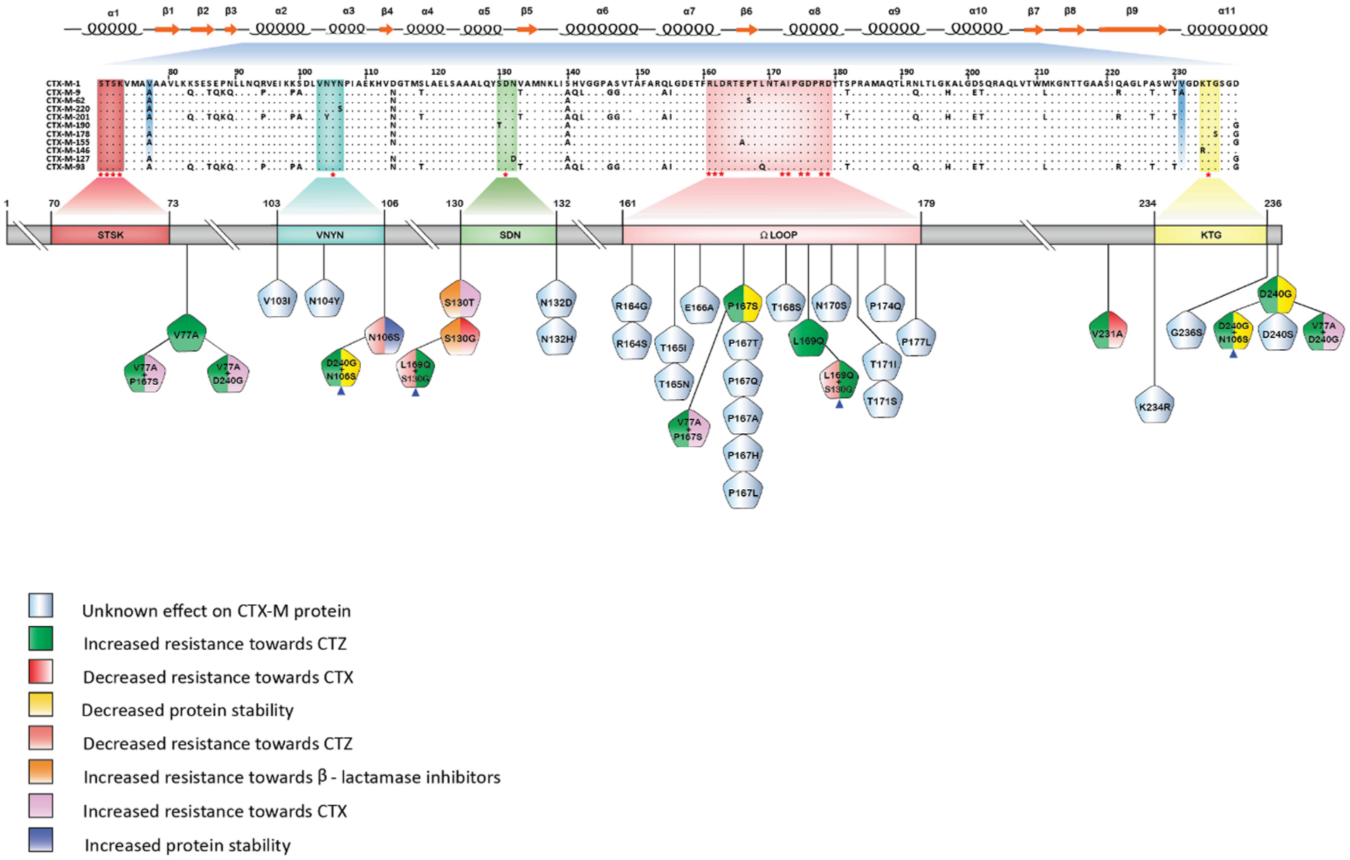

Fig. 3. Schematic representation of multiple sequence alignment of CTX-M sequences (n = 2210) showing detailed amino acid substitution. The red, blue, green and yellow boxes represent 70STSK73, 103VNYN106, 130SDN132 and 235KTG237 motif region respectively. The omega loop is indicated by pink box. The red star below the alignment represents the conserved amino acid residues. The mutations are represented in pentagonal-shaped polygon. The blue triangle below the pentagon indicates the in-vitro studied mutations. The effect of the mutations on CTX-M protein studied from the literature is represented by different colors. The plus sign in the pentagon indicates the combined mutations.

variants) followed by Asia (21 of 31 variants). Only one variant (D240G) was found in Africa. Australia contained only three different variations in CTX-M sequences namely: A77V, P167S and D240G. The D240G substitution was the maximum reported variation in all the continents. The spread of D240G substitution among different continents has been summarized in Table 1. Europe is also one of the hotspots for CTX-M containing bacteria and possess a total of 14 variants of CTX-M. South America possess only 6 variants (Fig. 4).

3.3. Spread patterns of CTX-M variations in India

There are geographical differences in the type of CTX-M genes found and there is scarcity of data from India. India contained only three different types of variations: D240G (maximum), E166A and V103I. In India, the CTX-M cases were reported in New Delhi, West Bengal, Karnataka and Tamil Nadu. The maximum reported cases are from New Delhi (n = 28) followed by South India (n = 22). Only D240G variation was found in West Bengal and Tamil Nadu. However, a new substitution (E166A) was found in Karnataka indicating that this variation is unique to this region (Fig. 5).

3.4. Selection Pressure

The nature of selection at each codon can feature the residues which interacts with β-lactam antibiotics. The four available important algorithms were used to investigate possible natural selection imparted on individual amino acid residue providing specific advantage to hydrolyze β-lactam ring more efficiently than the other. FEL infer dN/dS ratio at individual site by using maximum-likelihood approach [30]. It found one site under pervasive positive selection and 94 sites under purifying selection. Similarly, SLAC model which is also based on maximum-likelihood method along with the counting approach inferred positive selection at zero sites and purifying selection at 65 sites [30]. FUBAR is based on Bayesian approach and is more powerful than FEL when positive selection is present [31]. FUBAR identified 2 sites evolved under the influence of positive selection and 101 sites with the evidence of purifying selection. MEME model was used to investigate episodic positive selection at individual sites [32]. MEME inferred evidences of positive selection at 23 sites. SLAC detected zero sites under positive selection. Hence, the selection pressure analysis showed lower rate of positive selection and higher rate of negative selection in the motifs of CTX-M protein structure.

The focus was given to the amino acid residues contributing to the resistance towards cefotaxime and ceftazidime. The amino acid residues at position 77 (Val), 104 (Asn), 106 (Asn), 130 (Ser), 132 (Asn), 167 (Pro), 235(Lys), 237 (Gly) and 240 (Asp) were found to be under the influence of positive selection (dN/dS > 0) as detected by FEL, FUBAR and MEME model (Tables 2 and 3, Figs. 6 and 7).

Negative selection (dN/dS < 0) results in the conservation of the amino acid residues that does not undergo mutation under the influence of any external pressure. Apart from the differences of some amino acid residues, FEL, FUBAR, SLAC results were concordant in determining multiple sites under pervasive purifying selection. Many sites match with the conservative amino acid motifs and other critical residues necessary for the proper function of CTX-M protein, namely residues at position 70 (Ser), 72 (Ser), 73 (Lys), 105 (Tyr), 131 (Asp), 161 (Arg), 162

Table 1
The prevalence of different types of mutation in CTX-M among continents. Red font depicts the D240G mutation in each continent.

S. No.	Continents	Types of mutations	Number
1.	Africa	D240G	19
2.	Asia	D240G	517
		A77V	83
		V231A	7
		P167S	4
		R164G	1
		N132H	1
		P167L	2
		K234R	2
		P177L	3
		G236S	4
		V103I	44
		S130T	8
		N132D	2
		E166A	4
		P167A	2
		D240S	2
		T165N	1
		T168S	1
		T171S	2
		P167T	1
		P167Q	2
3.	Australia	A77V	3
		D240G	41
		P167S	5
4.	Europe	D240G	239
		A77V	162
		N132D	2
		K234R	3
		V231A	11
		P167S	7
		L169Q	2
		P167T	3
		T171S	3
		N106S	4
		V103I	8
		P174Q	2
		D240S	1
		T165I	1
5.	North America	A77V	85
		D240G	250
		V103I	14
		V231A	3
		P167T	4
		N132D	3
		D240S	3
		N170S	4
		K234R	5
		P167S	3
		T165N	1
		T168S	1
		P177L	1
		P167H	2
		N132H	2
		S130G	3
		S130T	6
		N106S	6
		N104Y	2
		T165I	1
		G236S	2
		R164S	2
		T171I	2
6.	South America	A77V	2
		V103I	8
		D240G	30
		V231A	2
		P167T	2
		P167S	1

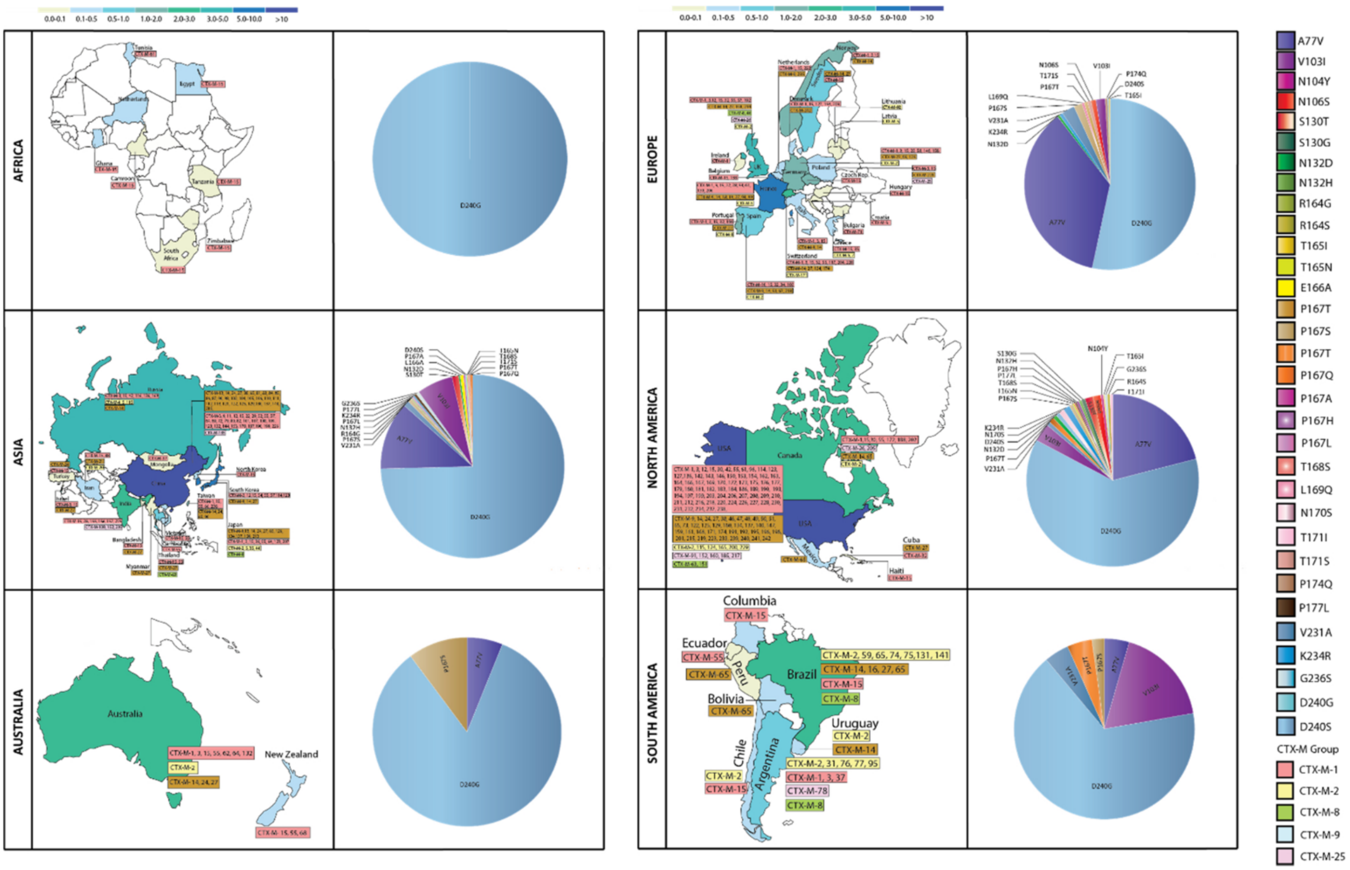

Fig. 4. Global distribution of CTX-M. Left panel represents the continent-wise (Africa, Asia, Australia, Europe, North America, South America) prevalence of CTX-M. The five CTX-M groups are depicted by different colors. Right panel depicts the distribution of amino acid variations of CTX-M protein found continent-wise shown through pie chart representation.

(Leu), 163 (Asp), 164 (Arg), 166 (Glu), 169 (Leu), 170 (Asn), 172 (Ala), 173 (Ile), 174 (Pro), 176 (Asp), 178 (Arg), 179 (Asp), 232 (Val) (Tables 2 and 3, Figs. 6 and 7).

4. Discussion

The CTX-M is a type of ESBL enzyme which specifically cleave the β-lactam ring (amide bond) present as a core molecule in the penicillin and cephalosporin antibiotics. The CTX-M enzymes present in the Gram-negative bacteria allow them to enjoy their pathogenesis in the presence of β-lactam. Although various CTX-M ESBL enzymes could hydrolyze the β-lactam ring. However, some selected amino acid residues in the specific region are found to vary in different CTX-M category with varying success rate in the environment as an infectious agent. The presence of selected amino acid in such critical region may be regarded as a result of natural selection/process. The selection of critical amino acid residue is an important factor to consider for the complexity of antibiotic resistance evolution. The D240G substitution is present in a number of CTX-M ESBL, indicating that it is important for the evolution of antibiotic resistance. This variant was reported globally suggesting a more aggressive trait allowing it to spread more quickly [33].

The amino acid residue at position 167, present in the omega loop (Ambler position 164–179) and Ambler position 240, present in beta-strand, were under the influence of strong positive selection. The Asp240 amino acid is situated at one end of β-3 strand and it is an important residue for substrate-binding pocket [10]. The P167S/T and D240G are favored by the bacteria and forms the most effective variants in hydrolyzing ceftazidime as it can accommodate its bulky R group by increasing the volume and flexibility of the active site [20]. These two sites have higher omega value (omega = dN/dS > 1), suggesting fast adaptation of the bacteria which might be due the presence of ceftazidime as strong selection pressure (Table 2) [20]. The substitution P167S is found in Asia, Europe, Australia and America. The P167S mutation alone produce a drastic loss of activity against cefotaxime, indicating that this change will only be favored in the environment containing ceftazidime [20]. The effect of P167S substitution was studied by molecular dynamics simulations of CTX-M-44 protein structure [10]. This revealed that the P167S substitution resulted in the altered positioning of aminothiazole ring due to the change in the movement of omega loop leading to ceftazidime hydrolysis [10]. *In vitro* analysis of P167S and L169Q artificial mutations in CTX-M-15 showed increased resistance against ceftazidime [34]. The D240G substitution is present in all the continents and is the only variation present in Africa. The D240G mutation resulted to enhanced hydrolytic activity against ceftazidime due to increased β-3 strand flexibility [10]. The P167S and D240G beneficial mutations never coexist in clinical isolates. *In vitro* study of P167S/D240G double mutant CTX-M-14 exhibit decreased ceftazidime hydrolysis and stability of enzyme [33,35].

The amino acid residue at position 130 present in the α helix was found to be positively selected residue. The diversified codon site S130G found in North America, has shown resistance against ceftazidime, demonstrating the potential selective force acting on this residue [34]. The S130T CTX-M mutants were found in Asia. Previous studies showed the effect of S130T substitution in novel CTX-M-199 and CTX-M-190 resulting in increased resistance towards β-lactamase inhibitors and at the same time maintained their hydrolytic activity on CTX [3,36]. These instances suggest that β-lactamase inhibitors might be the selective force acting on the residue S130 and has provided fitness advantages to bacteria. *In vitro* analysis of S130G mutation in CTX-M-9 carried out by Aumeran et al. in 2003 resulted in increased resistance towards

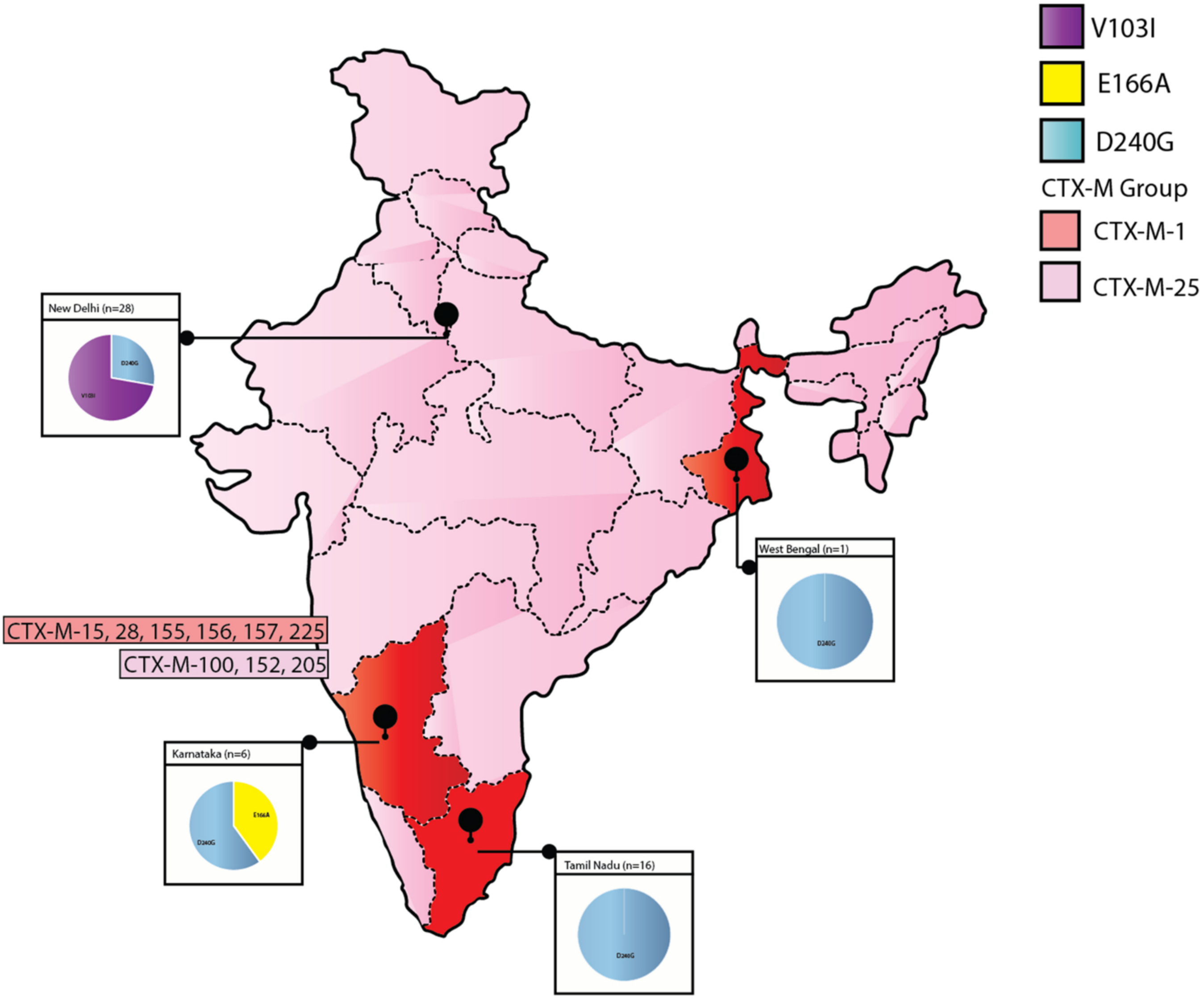

Fig. 5. Map showing distribution of CTX-M enzymes found in different states in India. The amino acid variations of CTX-M protein found state-wise is shown by pie chart representation.

Table 2
Positively Selected amino acid residues at site-level selection critical for β-lactam hydrolysis detected by HyPhy package (FEL, FUBAR, MEME) implemented in Datamonkey webserver.

Position	Amino Acid	FEL				FUBAR			MEME			
		α	β	LRT	p	α	β	β-α	α	β+	LRT	p+
77	Val	–	–	–	–	–	–	–	2.15	50.19	17.81	0.17
104	Asn	–	–	–	–	–	–	–	0.00	76.69	3.51	0.01
106	Asn	–	–	–	–	–	–	–	1.43	44.28	5.23	0.03
130	Ser	–	–	–	–	–	–	–	0.00	42.35	14.53	0.06
132	Asn	–	–	–	–	–	–	–	1.94	25.68	4.48	0.06
167	Pro	–	–	–	–	–	–	–	1.25	75.55	28.45	0.15
235	Lys	–	–	–	–	–	–	–	0.92	51.24	6.35	0.03
237	Gly	–	–	–	–	–	–	–	0.00	49.52	3.34	0.01
240	Asp	1.092	5.086	5.449	0.0196	1.206	13.054	11.848				

sulbactam and tazobactam but decreased hydrolytic activity against cefotaxime [37].

The residues at positions 77 (Val), 104 (Asn), 106 (Asn), 132 (Asn), 235(Lys), 237 (Gly) under the influence of positive selection have been mutated to A77V, N104Y, N106S, N132D/H, K234R, G235S. The A77V substitution is also prevalent and is found in all the continents except Africa. The positively selected residue at position 132 (N132D) present in CTX-M-215, found in Asia and North America, is highly resistant against mecillinum but not to cefotaxime, suggesting that this mutation has decreased the hydrolytic activity of CTX-M towards cephalosporin and hence, ceftazidime might not be the selective force acting on this residue [38]. The functional and structural role of N132H, K234R,

Table 3
Negatively selected amino acid residues at site-level selection critical for β-lactam hydrolysis detected by HyPhy package (FEL, FUBAR, SLAC) implemented in Datamonkey webserver.

Position	Amino Acid	FEL				SLAC			FUBAR		
		α	β	LRT	p	dN	dS	p	α	β	p
70	Ser	2.339	0.000	16.377	0.0001	0.00	7.46	0.00102	2.912	0.092	0.092
72	Ser	0.386	0.000	3.647	0.0562	–	–	–	1.077	0.128	0.938
73	Lys	0.596	0.000	0.117	6.285	0.00	3.43	0.0409	1.965	0.129	0.981
105	Tyr	3.217	0.000	18.012	18.012	0.00	7.41	0.00204	3.383	0.114	1.000
131	Asp	2.345	0.000	13.056	0.0003	0.00	5.10	0.00753	2.773	0.107	0.997
161	Arg	0.672	0.000	6.637	0.0100	0.00	2.85	0.0434	1.701	0.146	0.978
162	Leu	0.707	0.000	5.639	0.0176	0.00	5.14	0.00738	1.683	0.135	0.973
163	Asp	1.353	0.000	11.146	0.0008	1.00	5.04	0.0443	2.443	0.109	0.995
164	Arg	2.083	0.102	10.610	0.0011	–	–	–	3.323	0.343	0.997
166	Glu	1.510	0.083	6.705	0.0096	0.452	5.36	0.0280	3.108	0.339	0.985
169	Leu	1.371	0.106	6.830	0.0090	0.582	4.05	0.0501	2.702	0.325	0.992
170	Asn	1.633	0.152	5.784	0.0162	0.815	5.48	0.0453	2.364	0.497	0.938
172	Ala	0.604	0.000	5.301	0.0213	–	–	–	1.504	0.144	0.965
173	Ile	0.319	0.000	3.247	0.0716	–	–	–	1.002	0.123	0.937
174	Pro	1.320	0.103	6.262	0.0123	0.00	4.00	0.0123	2.648	0.109	0.998
176	Asp	1.500	0.000	11.533	0.0007	–	–	–	2.508	0.109	0.996
178	Arg	1.353	0.000	11.426	0.0007	–	–	–	–	–	–
179	Asp	0.425	0.000	3.644	0.0653	–	–	–	–	–	–
232	Val	1.035	0.099	4.698	0.0302	–	–	–	2.244	0.323	0.967

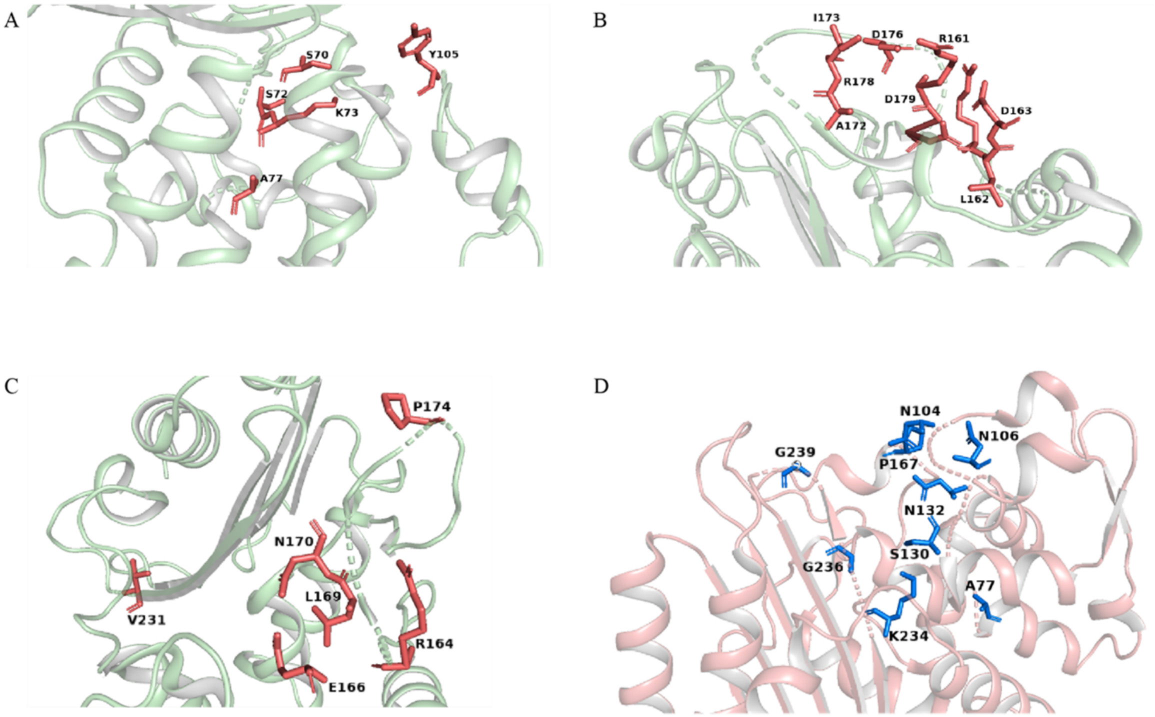

Fig. 6. Cartoon represents the amino acid residues under selection pressure demonstrated by Datamonkey models. The amino acid residues are shown by sticks. A, B and C represents the residues under negative selection. D represents the residues under positive selection.

G236S variable sites of CTX-M domain found in Asia and North America is presently unknown. Contrary to our study, the analysis did not show the residue at position 169 (L169Q) as the potential positively selected residue. However, L169Q mutation resulted in the enhancement of the CTX-M hydrolytic activity against ceftazidime [34].

There are various combination of substitutions occurring in nature including A77V/P167S, A77V/D240G. The A77V substitution increases the thermal stability of the CTX-M protein. The P167S and D240G mutation alone result in stability loss of CTX-M protein and decreased efficiency towards CTX which is compensated by the A77V variation.[29] *In vitro* study of double mutant N106S/D240G CTX-M-15 revealed the similar effect [39].

The residues at Ambler position 71, 73, 74 of the motif 70STSK74 are critical amino acids for the hydrolysis of β-lactam amide bond and are under strong negative selection. The Ser70 and one helix-turn downstream Lys73, both point at the bottom of the active site [27]. The Ser70 undergoes nucleophilic attack on the carbonyl carbon atom of β-lactam and interacts with deacylating water molecule [18]. Any change in these residues might downregulate the functioning CTX-M protein and hence, are selected by the nature. The amino acids at position 161 (Arg), 162 (Leu), 163 (Asp), 164 (Arg), 166 (Glu), 170 (Asn), 172 (Ala), 173 (Ile), 174 (Pro), 176 (Asp), 178 (Arg), 179 (Asp) falling under 19-residue long Ω loop (161–179), makes the floor of the active site and are under purifying selection. Although the negatively selected residues Glu166

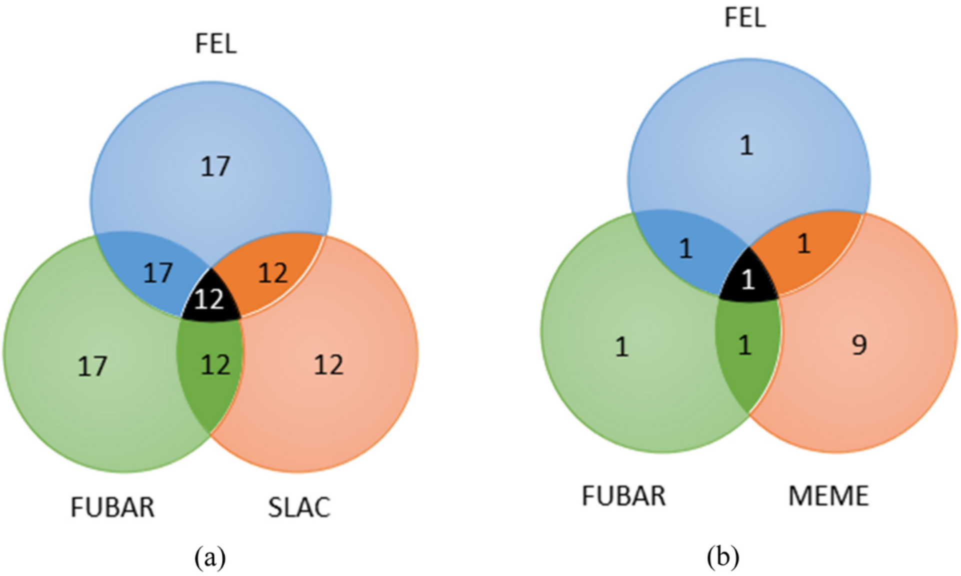

Fig. 7. Venn diagram showing the number of predicted sites under the influence of selection pressure by HyPhy method. (a) Positively selected amino acid sites detected by FEL, FUBAR, MEME (b) Negatively selected amino acid sites detected by FEL, FUBAR and SLAC.

and Asn170 plays an important role to place water molecule, W1 in close proximity to Ser70, E166A and N170S mutations have been seen in few CTX-M protein [40]. Despite being negatively selected amino acids by FEL, FUBAR, SLAC, there were variations detected in residues of Ω loop at position 164 (R164G/S), 169 (L169Q), 174 (P174Q). This might be due to the low frequency of occurrence of these mutation in the CTX-M sequences. By combining the data of selection pressure acting on amino acid residues with the variations found in continents, it captures a snapshot on the antibiotic usage in different countries.

Recent studies have shown that USA is one of the prolific user of antibiotics accounting for the maximum variations found in North America [41]. The study in Africa revealed the absence of accumulation of antimicrobial resistance (AMR) existence and thus elucidating the presence of only one variation (D240G) in the continent [42]. India and China are hotspots of antibiotic resistance in animals and there has been an increase in the third-generation cephalosporin consumption in humans explaining the high CTX-M variants found in Asia [41–43]. According to our study, there are high number of cases reported in South India, which corroborate with the previous reports [44,45]. Only D240G and E166A variations were found in Southern parts of India which is contradictory to the study carried by Vaiyapuri et al., which also showed the occurrence of A77V substitution [46]. Another study has reported the presence of D240G substitution in Maharashtra [47].

5. Conclusion

Our study provides a novel perspective on the distribution pattern of CTX-M amino acid variations globally. The D240G substitution was predominantly found in all the continents that confers increase in resistance against ceftazidime. Along with D240, the A77 and P167 amino acid positions were found under the influence of positive selection favoring bacterial survival. On account of the above study, the gradual decrease in the effectiveness of the antibiotics is the threat to public health globally. Thus, significant action and major policies including balanced use of antibiotics and proper sanitization must be applied.

Funding

The authors gratefully acknowledged Science and Engineering Research Board (SERB), Department of Science and Technology, Government of India for funding the project (Grant number: EMR/2017/004602).

Conflict of Interest

There is no conflict of interest. All authors read and approved the current submitted version of manuscript to Comparative Immunology, Microbiology and Infectious Diseases.

Acknowledgements

We would like to express our sincere gratitude to Dr. M. S. Chauhan, Director, ICAR-National Dairy Research Institute, Karnal - 132001, Haryana, India for the necessary support and assistance during the study.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.cimid.2022.101815.

References

[1] R.P. Veiga, J.-A. Paiva, Pharmacokinetics-pharmacodynamics issues relevant for the clinical use of beta-lactam antibiotics in critically ill patients 11 Medical and Health Sciences 1103 Clinical Sciences, Critical Care (2018).
[2] Foster_et_al_2019.pdf, (n.d.).
[3] Q. Cheng, C. Xu, J. Chai, R. Zhang, E. Wai Chi Chan, S. Chen, Structural Insight into the Mechanism of Inhibitor Resistance in CTX-M-199, a CTX-M-64 Variant Carrying the S130T Substitution, ACS Infect. Dis. 6 (2020) 577–587, https://doi.org/10.1021/acsinfecdis.9b00345.
[4] C.L. Tooke, P. Hinchliffe, E.C. Bragginton, C.K. Colenso, V.H.A. Hirvonen, Y. Takebayashi, J. Spencer, β-Lactamases and β-Lactamase Inhibitors in the 21st Century, J. Mol. Biol. 431 (2019) 3472–3500, https://doi.org/10.1016/j.jmb.2019.04.002.
[5] Betaâ_Lactams _ Enhanced Reader, (n.d.).
[6] C.J.H. Von Wintersdorff, J. Penders, J.M. Van Niekerk, N.D. Mills, S. Majumder, L. B. Van Alphen, P.H.M. Savelkoul, P.F.G. Wolffs, Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer, Front. Microbiol. 7 (2016) 1–10, https://doi.org/10.3389/fmicb.2016.00173.
[7] C. Brandt, S.D. Braun, C. Stein, P. Slickers, R. Ehricht, M.W. Pletz, O. Makarewicz, In silico serine β-lactamases analysis reveals a huge potential resistome in environmental and pathogenic species, Sci. Rep. 7 (2017) 1–13, https://doi.org/10.1038/srep43232.
[8] K. Bush, G.A. Jacoby, A.A. Medeiros, MINIREVIEW A Functional Classification Scheme for-Lactamases and Its Correlation with Molecular Structure, 1995.

[9] R.P. Ambler, The structure of beta-lactamases, Philos. Trans. R. Soc. Lond. B. Biol. Sci. 289 (1980) 321–331, https://doi.org/10.1098/rstb.1980.0049.

[10] T. Palzkill, Structural and mechanistic basis for extended-spectrum drug-resistance mutations in altering the specificity of TEM, CTX-M, and KPC β-lactamases, Front. Mol. Biosci. 5 (2018) 1–19, https://doi.org/10.3389/fmolb.2018.00016.

[11] A. Boyd, S. HIV Monitoring, N. Bao-Tao Liu, J. Cai, J. Chen, S. Chen, Y. Jiang, R. Zhang, Fecal carriage and genetic characterization of CTX-M-1/9/1-producing escherichia coli from healthy humans in Hangzhou, China (2021), https://doi.org/10.3389/fmicb.2021.616687.

[12] H. Lahlaoui, A. Ben Haj Khalifa, M. Ben Moussa, Epidemiology of Enterobacteriaceae producing CTX-M type extended spectrum β-lactamase (ESBL), Med. Mal. Infect. 44 (2014) 400–404, https://doi.org/10.1016/j.medmal.2014.03.010.

[13] W.H. Zhao, Z.Q. Hu, Epidemiology and genetics of CTX-M extended-spectrum β-lactamases in Gram-negative bacteria, Crit. Rev. Microbiol. 39 (2013) 79–101, https://doi.org/10.3109/1040841X.2012.691460.

[14] R. Cantón, J.M. González-Alba, J.C. Galán, CTX-M enzymes: origin and diffusion, Front. Microbiol. 3 (2012), https://doi.org/10.3389/fmicb.2012.00110.

[15] E. Soto, N. Espinosa, crossm Functional Characterization of EscK, 199 (2017) 1–19.

[16] Y. Chong, S. Shimoda, N. Shimono, Current epidemiology, genetic evolution and clinical impact of extended-spectrum β-lactamase-producing Escherichia coli and Klebsiella pneumoniae, Infect. Genet. Evol. 61 (2018) 185–188, https://doi.org/10.1016/j.meegid.2018.04.005.

[17] A. Karim, L. Poirel, S. Nagarajan, P. Nordmann, Plasmid-mediated extended-spectrum Î^{2}-lactamase (CTX-M-3 like) from India and gene association with insertion sequence ISEcp1, FEMS Microbiol. Lett. 201 (2006) 237–241, https://doi.org/10.1111/j.1574-6968.2001.tb10762.x.

[18] A. Philippon, P. Slama, P. Dény, R. Labia, A structure-based classification of class A β-Lactamases, a broadly diverse family of enzymes, Clin. Microbiol. Rev. 29 (2016) 29–57, https://doi.org/10.1128/CMR.00019-15.

[19] F. José Pérez-Llarena, F. Kerff, O. Abián, S. Mallo, M.C. Fernández, M. Galleni, J. Sancho, G. Bou, Distant and new mutations in CTX-M-1-lactamase affect cefotaxime hydrolysis, Antimicrob. Agents Chemother. 55 (2011) 4361–4368, https://doi.org/10.1128/AAC.00298-11.

[20] Â. Novais, I. Comas, F. Baquero, R. Cantón, T.M. Coque, A. Moya, F.G. González-Candelas, J.C. Galán, Evolutionary trajectories of beta-lactamase CTX-M-1 cluster enzymes: Predicting antibiotic resistance, PLoS Pathog. 6 (2010), https://doi.org/10.1371/journal.ppat.1000735.

[21] MEGA-X.pdf, (n.d.).

[22] M.M. D'Andrea, F. Arena, L. Pallecchi, G.M. Rossolini, CTX-M-type β-lactamases: a successful story of antibiotic resistance, Int. J. Med. Microbiol. 303 (2013) 305–317, https://doi.org/10.1016/j.ijmm.2013.02.008.

[23] D. He, S.R. Partridge, J. Shen, Z. Zeng, L. Liu, L. Rao, L. Lv, J.H. Liu, CTX-M-123, a novel hybrid of the CTX-M-1 and CTX-M-9 group β-lactamases recovered from Escherichia coli isolates in china, Antimicrob. Agents Chemother. 57 (2013) 4068–4071, https://doi.org/10.1128/AAC.00541-13.

[24] Q. Cheng, C. Xu, J. Chai, R. Zhang, E. Wai Chi Chan, S. Chen, Structural insight into the mechanism of inhibitor resistance in CTX-M-199, a CTX-M-64 variant carrying the S130T substitution, ACS Infect. Dis. 6 (2020) 577–587, https://doi.org/10.1021/acsinfecdis.9b00345.

[25] G.B. Tian, Y.M. Huang, Z.L. Fang, Y. Qing, X.F. Zhang, X. Huang, CTX-M-137, a hybrid of CTX-M-14-like and CTX-M-15-like β-lactamases identified in an Escherichia coli clinical isolate, J. Antimicrob. Chemother. 69 (2014) 2081–2085, https://doi.org/10.1093/jac/dku126.

[26] F.J. Pérez-Llarena, F. Kerff, O. Abián, S. Mallo, M.C. Fernández, M. Galleni, J. Sancho, G. Bou, Distant and new mutations in CTX-M-1 β-lactamase affect cefotaxime hydrolysis, Antimicrob. Agents Chemother. 55 (2011) 4361–4368, https://doi.org/10.1128/AAC.00298-11.

[27] J. Delmas, Y. Chen, F. Prati, F. Robin, B.K. Shoichet, R. Bonnet, Structure and dynamics of CTX-M enzymes reveal insights into substrate accommodation by extended-spectrum β-Lactamases, J. Mol. Biol. 375 (2008) 192–201, https://doi.org/10.1016/j.jmb.2007.10.026.

[28] Y. Chen, J. Delmas, J. Sirot, B. Shoichet, R. Bonnet, Atomic resolution structures of CTX-M β-lactamases: Extended spectrum activities from increased mobility and decreased stability, J. Mol. Biol. 348 (2005) 349–362, https://doi.org/10.1016/j.jmb.2005.02.010.

[29] M.P. Patel, B.G. Fryszczyn, T. Palzkill, Characterization of the global stabilizing substitution A77V and its role in the evolution of CTX-M β-Lactamases, Antimicrob. Agents Chemother. 59 (2015) 6741–6748, https://doi.org/10.1128/AAC.00618-15.

[30] M. Marinić, V.J. Lynch, Relaxed constraint and functional divergence of the progesterone receptor (PGR) in the human stem-lineage, PLoS Genet. 16 (2020) 1–19, https://doi.org/10.1371/journal.pgen.1008666.

[31] S. Kumar, J.S. Chera, A. Vats, S. De, Nature of selection varies on different domains of IFI16-like PYHIN genes in ruminants, BMC Evol. Biol. 19 (2019) 1–11, https://doi.org/10.1186/s12862-018-1334-7.

[32] Drosophila Academy of Sci, n.d.

[33] C.A. Brown, L. Hu, Z. Sun, M.P. Patel, S. Singh, J.R. Porter, B. Sankaran, B. V. Venkataram Prasad, G.R. Bowman, T. Palzkill, Antagonism between substitutions in β-lactamase explains a path not taken in the evolution of bacterial drug resistance, J. Biol. Chem. 295 (2020) 7376–7390, https://doi.org/10.1074/jbc.RA119.012489.

[34] F. Compain, M. Arthur, crossm Combination of Amino Acid Substitutions Leading to CTX-M-, 62 (2018) 1–8.

[35] C.M. Miton, K. Buda, N. Tokuriki, Epistasis and intramolecular networks in protein evolution, Curr. Opin. Struct. Biol. 69 (2021) 160–168, https://doi.org/10.1016/j.sbi.2021.04.007.

[36] Z. Shen, B. Ding, Y. Bi, S. Wu, S. Xu, X. Xu, Q. Guo, M. Wang, crossm 61 (2017) 1–5.

[37] C. Aumeran, C. Chanal, R. Labia, D. Sirot, J. Sirot, R. Bonnet, Effects of Ser130Gly and Asp240Lys substitutions in extended-spectrum-lactamase CTX-M-9, Antimicrob. Agents Chemother. 47 (2003) 2958–2961, https://doi.org/10.1128/AAC.47.9.2958-2961.2003.

[38] I. Vivo, N. Narrow-spectrum, M. Yin, G. Hu, Z. Shen, C. Fang, X. Zhang, D. Li, Y. Doi, Y. Zhang, crossm Resistance to Mecillinam, 64 (2020) 1–6.

[39] Â. Novais, R. Cantón, T.M. Coque, A. Moya, F. Baquero, J.C. Galán, Mutational events in cefotaximase extended-spectrum β-lactamases of the CTX-M-1 cluster involved in ceftazidime resistance, Antimicrob. Agents Chemother. 52 (2008) 2377–2382, https://doi.org/10.1128/AAC.01658-07.

[40] P.D. Kilmer, Review article: review article, Journalism 11 (2010) 369–373, https://doi.org/10.1177/1461444810365020.

[41] M.A.T. Blaskovich, The fight against antimicrobial resistance is confounded by a global increase in antibiotic usage, ACS Infect. Dis. 4 (2018) 868–870, https://doi.org/10.1021/acsinfecdis.8b00109.

[42] T.P. Van Boeckel, J. Pires, R. Silvester, C. Zhao, J. Song, N.G. Criscuolo, M. Gilbert, S. Bonhoeffer, R. Laxminarayan, Global trends in antimicrobial resistance in animals in low- and middle-income countries, Science (80-.) 365 (2019), https://doi.org/10.1126/science.aaw1944.

[43] S. Gandra, J. Joshi, A. Trett, A. Sankhil Lamkang, Suggested citation, 2017.

[44] R. Nachimuthu, V.R. Kannan, B. Bozdogan, V. Krishnakumar, K.P. S, P. Manohar, CTX-M-type ESBL-mediated resistance to third-generation cephalosporins and conjugative transfer of resistance in Gram-negative bacteria isolated from hospitals in Tamil Nadu, India, Access Microbiol. 3 (2021), https://doi.org/10.1099/acmi.0.000142.

[45] A. Aggarwal, S. Mehta, D. Gupta, S. Sheikh, S. Pallagatti, R. Singh, I. Singla, Clinical & immunological erythematosus patients characteristics in systemic lupus Maryam, J. Dent. Educ. 76 (2012) 1532–1539, https://doi.org/10.4103/ijmr.IJMR.

[46] M. Vaiyapuri, A.S.P. Sebastian, I. George, S.S. Variem, R.N. Vasudevan, J. C. George, M.R. Badireddy, V. Sivam, S. Peeralil, D. Sanjeev, M. Thandapani, S. A. Moses, R.C. Nagarajarao, M.P. Mothadaka, Predominance of genetically diverse ESBL Escherichia coli identified in resistance mapping of Vembanad Lake, the largest fresh-cum-brackishwater lake of India, Environ. Sci. Pollut. Res. 28 (2021) 66206–66222, https://doi.org/10.1007/s11356-021-15110-y.

[47] R. Dhawde, R. Macaden, D. Saranath, K. Nilgiriwala, A. Ghadge, T. Birdi, Antibiotic Resistance Characterization of Environmental E. coli Isolated from River Mula-Mutha, Pune District, India, (n.d.). https://doi.org/10.3390/ijerph15061247.