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Studies on structure-based sequence alignment and phylogenies of beta-lactamases

Parveen Salahuddin & Asad U Khan*

Distributed Information Sub-Centre, Interdisciplinary Biotechnology Unit, A. M. U. Aligarh, 202002, India; Asad U Khan – Email: asad.k@rediffmail.com; Phone: +91 571 2723088; Fax: +91 571 2721776; *Corresponding author

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Abstract:
The β-lactamases enzymes cleave the amide bond in β-lactam ring, rendering β-lactam antibiotics harmless to bacteria. In this communication we have studied structure-function relationship and phylogenies of class A, B and D beta-lactamases using structure-based sequence alignment and phylip programs respectively. The data of structure-based sequence alignment suggests that in different isolates of TEM-1, mutations did not occur at or near sequence motifs. Since deletions are reported to be lethal to structure and function of enzyme. Therefore, in these variants antibiotic hydrolysis profile and specificity will be affected. The alignment data of class A enzyme SHV-1, CTX-M-15, class D enzyme, OXA-10, and class B enzyme VIM-2 and SIM-1 show sequence motifs along with other part of polypeptide are essentially conserved. These results imply that conformations of beta-lactamases are close to native state and possess normal hydrolytic activities towards beta-lactam antibiotics. However, class B enzyme such as IMP-1 and NDM-1 are less conserved than other class A and D studied here because mutation and deletions occurred at critically important region such as active site. Therefore, the structure of these beta-lactamases will be altered and antibiotic hydrolysis profile will be affected. Phylogenetic studies suggest that class A and D beta-lactamases including TOHO-1 and OXA-10 respectively evolved by horizontal gene transfer (HGT) whereas other member of class A such as TEM-1 evolved by gene duplication mechanism. Taken together, these studies justify structure-function relationship of beta-lactamases and phylogenetic studies suggest these enzymes evolved by different mechanisms.

Background:
Antibiotics are widely used to treat bacterial infections. However, bacteria have now become increasingly resistant to conventional antibiotics resulting in a major issue in clinical settings.

Antibiotic resistance in bacterial strains have disseminated widely and posed a great threat to public health. β-lactam antibiotics are the most commonly prescribed drugs as the mainstay for the treatment of many bacterial infections. These antibiotics contain β-lactam ring and inhibit synthesis of peptidoglycan layer of bacterial cell wall **[1].** However, bacteria have developed several strategies to resist antibiotic action among them the most effective is the production of β-lactamases which catalyze hydrolysis of β-lactam antibiotics **[2]** thereby opening the β-lactam ring and rendering it inactive **[3].** In addition, administration of β-lactamase inhibitor, such as clavulanic acid, together with β-lactam antibiotic is an effective method to treat β-lactamase producing infections.The latter strategy effectively blocks β-lactamase activity while the β-lactam antibiotic is free to inhibit the bacterial transpeptidases.

Classification of β-lactamases have been based on either primary structure such as conserved amino acids and protein sequence motifs **[4]** or the functional characteristics of the enzymes **[5, 6].** The simplest classification scheme depends upon protein structure, whereby the β-lactamases are classified into four molecular classes, A, B, C, and D **[4, 7-9].** The structural approach is the easiest way to classify these enzymes. Ambler originally proposed two classes:class A, the active-site serine β-lactamases; and class B, the metallo-β-lactamases that require a bivalent metal ion, usually Zn^{2+}, for activity. Shortly

after, a new class of serine β-lactamase was found that bored little sequence similarity to the then-known class A enzymes. Designated as class C, its members are also known as the 'AmpC' β-lactamases. Finally, another class of serine β-lactamases such as OXA β-lactamases, was discovered that bear little resemblance to either class A or class C and was designated as class D. These A, C, D classes of enzymes show sufficient structural homology indicating that they might have arosed from a common ancestor **[10].** Class B consists of metallo beta-lactamases and is perhaps the most heterogeneous class among all of the classes of beta-lactamases. This class of enzyme has been further divided into a number of sub-classes including B1, B2 and B3 [**10, 11].**

Horizontal gene transfer and duplication are some of the most common mechanisms of evolution by which beta-lactamases genes have evolved. Horizontal gene transfer event can be detected by incongruency between gene tree and species tree and duplication can be determined provided homologous sequences from the same species occupy the same clade in a phylogenetic tree.

Structure-based sequence alignment of proteins can provide a wealth of information on structure-function relationship **[12].** Using Clustal-O and ESPript 2.2 programs **[13, 14]** we have constructed and displayed structure-based sequence alignment of class A, B and D beta-lactamases and thereby we have determined structure- function relationships. We have also analyzed effect of deletion of signal peptide on secretion process. Studies on class C enzyme namely AmpC was omitted because of heterogeneity in data due to the presence of several long insertions and deletions of amino acids. The phylogenetic trees of class A, B and D beta-lactamases were constructed using Phylip 2.37 **[15]** and origin of resistant gene and mechanism of evolution were determined.

Methodology:

Structure-based sequence alignment

The sequences of different beta-lactamases were retrieved from NCBI (http://www.ncbi.nlm.nih.gov). The sequences of beta-lactamases selectd for this study are aligned using Clustal O **[13, 14]** and displayed using ESPript 2.2 **[12]** (**Figures & Supplementary Materials**).

Phylogenetic Tree

Alignments of the selected sequences of beta-lactamase were performed by Clustal-Omega: multiple sequence alignment program with default parameters **[13, 14].** Phylogenetic tree was constructed using Phylip 2.37 program **[15].** Distance matrix from protein sequences was constructed using phylip 3.67: protdist program. Phylogenetic tree was built by Neighbour joining **[16]** and was drawn by Phylowidget program **[17].** Bootstrap was set to 1000 and bootstrap value of all internal nodes of all phylogenetic trees were obtained by performing computation in the following sequence of order using phylip programs **[15]:** Seqboot→Protdist→Neighbour Joining Consensus. Values of bootsrap from consensus tree were manually plotted on to phylogenetic tree. The species tree was builded using server http://www. ncbi.nlm.nih. gov/ nTaxonomy/CommonTree/www.cmt.cgi). Finally, species tree was opened and saved using phylodendron server (http://iubio.bio.indiana.edu/treeapp/). Cases of horizontal gene transfer were inferred by comparing species tree and gene tree and incongruency in the data suggest occurrence of horizontal gene transfer.

Results & Discussion:

Structure-function studies of class A beta-lactamases

We have retreived those sequences of beta-lactamases from NCBI that shows 85% to 100% conservation. Thereafter, we have constructed and displayed structure-based sequence alignment of beta lactamases using Clustal O and ESPript2.2 programs **[13, 14, 12].** The program ESPript, Easy Sequencing in PostScript, generate figures of aligned sequences with secondary structure information. ESPript reads text outputs from multiple sequence alignment programs such as Clustal O **[13, 14].** Residues are boxed according to their similarity score and secondary structure elements are adorned at the top of sequences blocks. From structure-based sequence alignment the effect of mutations or indels on structural integrity of beta-lactamase was determined and from these data their function was inferred.

The TEM-1 enzyme is the best known member of the class A β-lactamases **[18, 19],** that efficiently hydrolyzes β-lactam antibiotics, particularly penicillins, and thus provides a mechanism of resistance against them. The structure-based sequence alignment data of TEM-1 is depicted in **Figure S1, S2, S3 (Available with authors)** and is found to be 85.31% conserved. Inspection of four conserved motifs, S-X-X-K, S-X-N, K-T/S-G, and the Ω-loop commonly found in the vicinity of the active-site pocket of TEM-1 isolates **[19]** suggest hundred percent conservation. However, ABD61074 and YP_001966135 variants of TEM-1 bored Phe to Leu and Ala to Ser mutations at signal sequence. Similarly, ADL40245 variant contains hydrophobic Ala to polar Ser mutations at the signal sequence. These substitutions may affect secretion process of these variants of TEM-1. Additionally in four isolates Met1 was deleted from signal sequence which might also affect secretion process. Besides this, in several variants part of α-helix 8 is missing whereas in other isolates entire α-helix 8 and β-sheet 9 segments were deleted from the C-terminus. Recent studies have shown that enzyme TEM-1 β-lactamase was broadly tolerant to the deletion mutations sampled. Infact variants analyzed retained activity towards ampicillin, with deletion mutations observed in helices and strands as well as regions important for structure and function **[20].** But in our analysis we found an entire helical segment as well as β-sheet deleted. Therefore, it is highly likely that catalytic efficiency and substrate hydrolysis profile of these variants of TEM-1 will be affected.

The SHV-1enzyme belongs to the molecular class A serine β-lactamases and share extensive structural and functional similarity with TEM β-lactamases. The SHV-1 β-lactamase behaves as a typical penicillinase hydrolyzing penicillins and early generation cephalosporins.The SHV-1 beta lactamase was found to be 90.56 % conserved. Like TEM-1 enzyme, SHV-1 enzyme did not harbored mutations at or near sequence motifs S-X-X-K, S-D-N and K-T-G and Ω-loop **(Figure 1).** Rest of the part of polypeptide of most of the SHV-1 variants was found to be fully conserved. However, our data indicate that AAF34333 variant contains Leu 170 →Phe170 substitution and lacks signal peptide sequence and part of the C- terminal helical structure.

Due to deletion of signal peptide, this variant of SHV-1 will be secreted but because of deletion of C-terminal helix and mutation the structure of the variant is altered since deletion mutations are considered to be lethal to structure and function of proteins **[21, 22].**

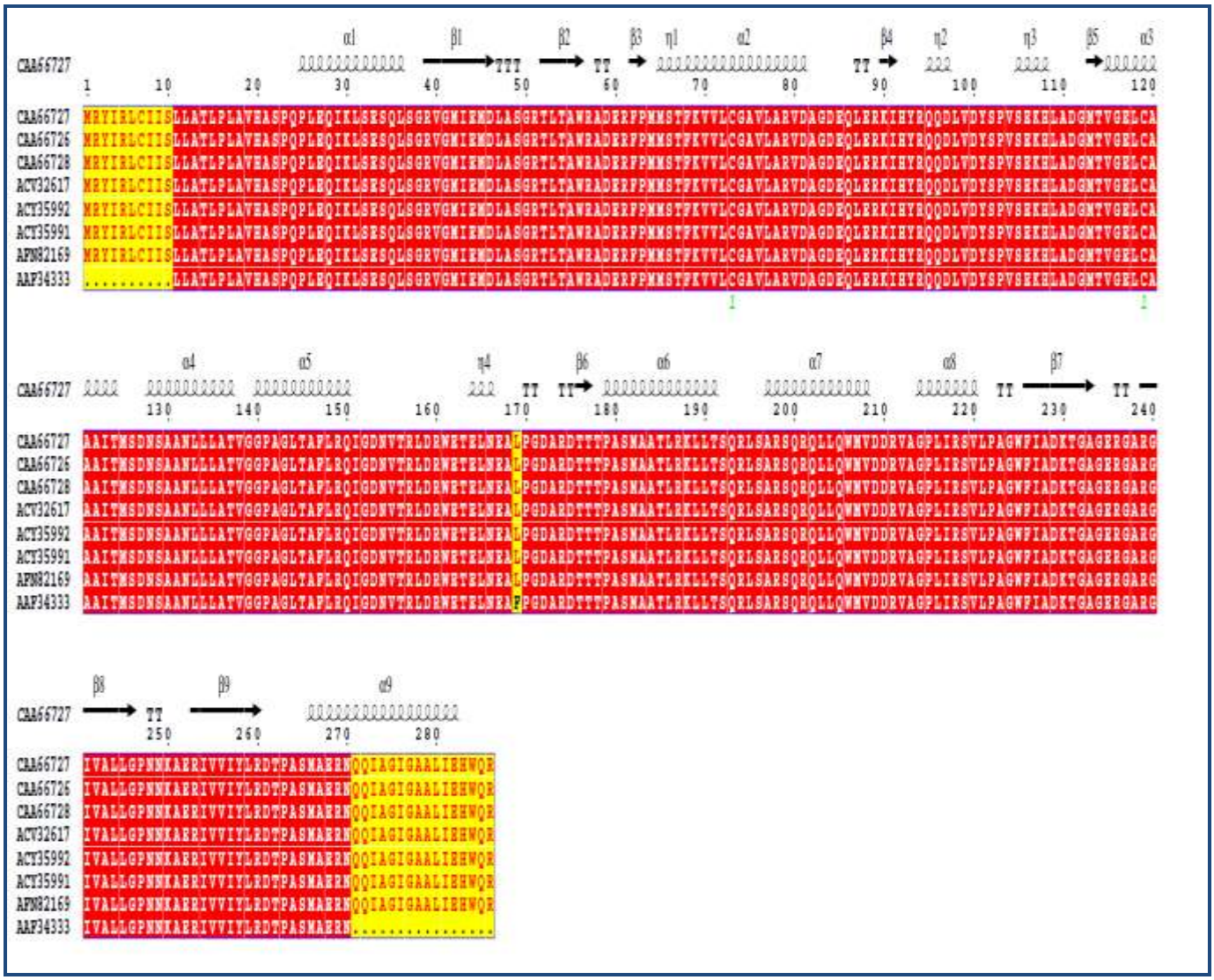

Figure 1: Structure-based sequence alignment of SHV-1 variants.

CTX-M enzyme (derived its name from being highly active on CefoTaXime and isolated in Munich) exhibits greater hydrolytic activity against cefotaxime in comparison to ceftazidime **[23].** However, it has been reported that some clinical isolates show a significant degree of resistance towards ceftazidime as well **[24].** The (**Figure 2)** contain structure-based sequence alignment data of CTX-M-15 and its homologue TOHO-1 respectively. The alignment data of CTX-M-15 and TOHO-1 suggest 93.25 % and 100 % conservations. Analyses of CTX-M-15 alignment file indicate motif sequences are essentially conserved (shown in boxes) (**Figure 2a**). In several variants of CTX-M-15 signal sequence is absent while in other variants it is present. Those variants lacking signal sequence will be secreted. Upon close observation we found that AFN068021 variant of CTX-M-15 from *Escherechia Coli* bore only one mutation near motif sequence. However, that mutation was found to be conservative. Hence, it will not affect the structure-function of the enzyme. Similarly, inspection of alignment data of TOHO-1 **(Figure 2b),** a close homologue of CTX-M-15, suggests 100% conservation. Therefore, these variants of TOHO-1 possess native-like structure and normal hydrolytic activity towards beta-lactam antibiotics.

Structure-function studies of class D beta-lactamases

OXA-10 beta-lactamase is so named because of avid hydrolytic activity of oxacillin. It lacks overall sequence similarity to class A enzyme and confers high levels of resistance to broad spectrum of antibiotics **[5].** Examination of alignment file suggests 98.87% conservation. The sequence motifs (shown in boxes) **Figure S2 (Available with author**) are strictly conserved in OXA-10 and among class D enzymes **[25].** However, B9W1Z5 variant harbored conservative mutation Ala111 to Val111 in α-helix 4 and non-conservative mutation Asn216 to Ser 216 in an unstructured region which will not alter structure and function of the enzyme. Additionally, the B6D9D4 variant bored non-conservative mutation where Lys138 was mutated to Asn138 at the end of alpha helix 5. Since Asn residue is present at the beginning and end of the helix. Its role can be thought as

"capping" the hydrogen bond interactions. Therefore, this mutation may stabilize the structure of the enzyme. This result is supported by the findings that alpha-helical structure is stabilized by end capping **[26].**

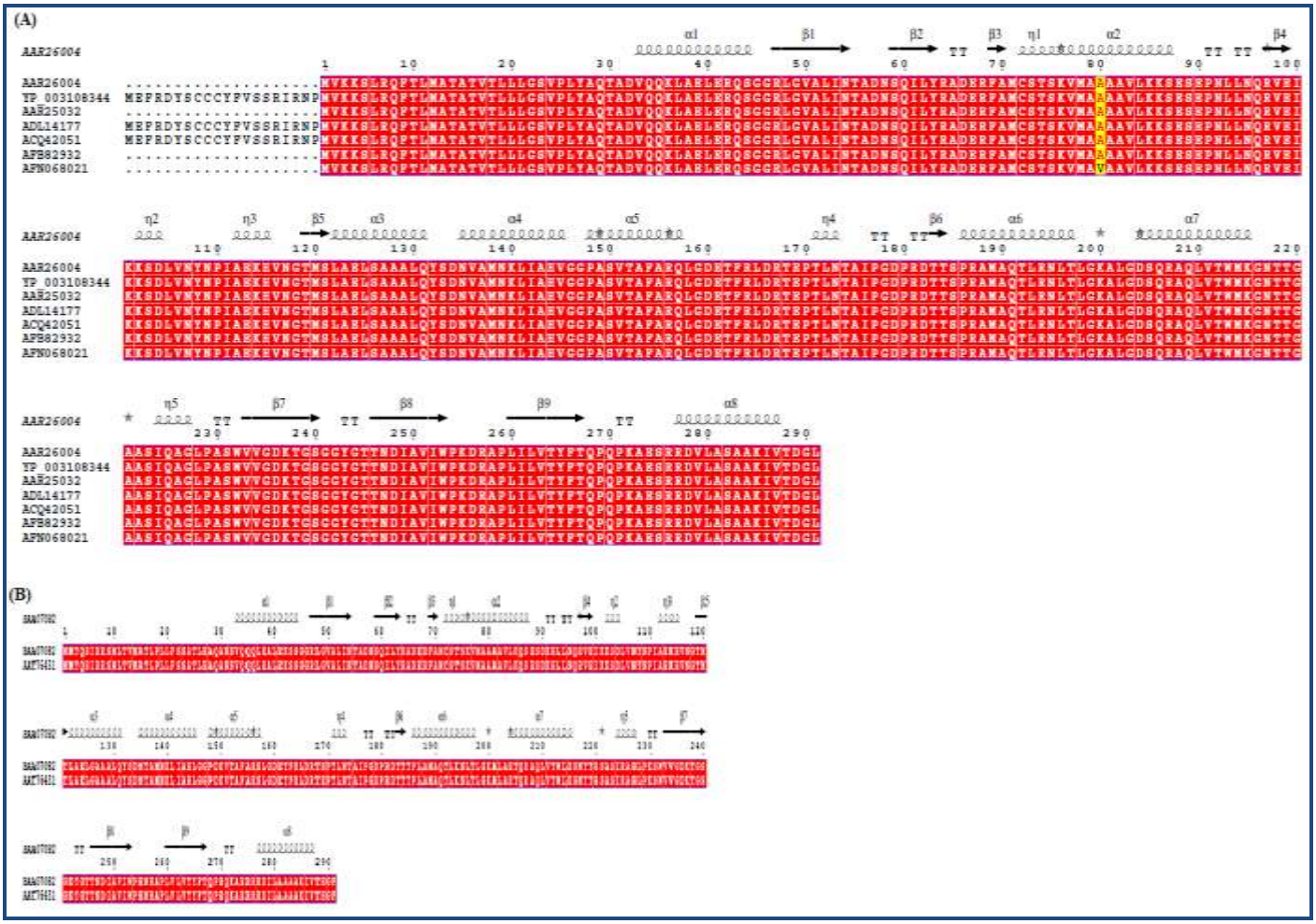

Figure 2: A) Structure-based sequence alignment of CTX-M-15 variants; **B)** Structure-based sequence alignment of TOHO-1 variants.

Structure- function studies of class B beta lactamases

Metallo-beta-lactamases are based on functional characteristics and are classified as group 3 enzyme because they hydrolyze penicillin, cephalosporins and carbapenems but are resistant to almost all conventional beta-lactam inhibitors. According to ambler classification scheme they are classified as molecular class B enzymes. Despite low sequence similarity between various metallo-beta-lactamases, the general tertiary structure of these enzymes is very similar. The alignment file of VIM-2 and SIM-1 showed 100% conservation (**Figure S3 & Figure S4 (Available with author)**. Therefore, these beta-lactamases possess native like structure with normal catalytic activity. Analysis of structure-based sequence alignment of IMP suggests 95.93% conservation. One variant such as CAK55562 harbored Arg76Pro and Val155Leu substitutions. First substitution Arg76Pro signifies the termination of helix-1 where as second mutation increases the hydrophobicity of β-sheet 10. Thus, overall structure of this variant is stabilized. Further, the structure of IMP-1 indicates that zinc ion (Zn1) is coordinated by His116, His118, and His196, and second zinc (Zn2) is coordinated by residues Asp120, Cys221, and His263 **[10].** Upon further analysis we found that catalytic residues such as His116, His 118, Asp120 and K224 are conserved **Figure S5 (Available with author).** But in all isolates of IMP-1 substitutions occurred at other positions critical for the catalytic activity including His196Ser and Cys221Ser. Hence the geometry of the active site is altered and hydrolysis profile and specificity towards antibiotics is affected **[27].**

NDM-1 showed lower sequence identity with other metallo beta-lactamases, and the most closely related metallo beta-lactamases are VIM-2 and IMP-1 with which it shows 32% sequence identity. The structure-based sequence alignment data indicate 91.48% conservation. NDM-1 contains an additional insert between positions 162 and 166 not present in other MBLs. The active site of NDM-1 is located at the bottom of a shallow groove enclosed by 2 important loops, L3 and L10 **[28].** Variant such as AEQ39156 possess Gly178 to Ser178 substitution in the turn segment which will restrict flexibility of this region. Furthermore, the C terminus fragment containing catalytic His 250 is also absent. Thus, structure and catalytic activity of this variant is altered. Besides this, in other variants, an unstructured C-terminus region containing catalytic His 250 and α-helix-6 were absent. Therefore, in these variants of NDM-1 antibiotic hydrolysis and specificity will be profoundly affected. These findings are validated by mutagenesis studies

that showed mutation of active site residues of NDM-1 resulted in loss after catalytic activity **[29].** Thus, like IMP, NDM-1 is less conserved **Figure S6 (Available with author).** Our data clearly indicate that class B enzyme such as IMP-1 and NDM-1 are less conserved than VIM2 and SIM-1 studied here because mutation occurred at the critically important region such as active site. Thus, A, B and D classess of beta-lactamases showed structure-function relationship. Additionally, one important aspect of our data is deletions and active site mutations of amino acids have leaded to loss of beta-lactamases structures and functions. Since indel mutations and active site mutations are subjected to purifying selection in coding regions **[30].** Therefore, these beta-lactamases lactamases are subjected to purifying selection.

Phylogenetic tree analysis of class A beta-lactamases

Phylogenetic tree or evolutionary tree is a branching diagram or "tree" showing the inferred evolutionary relationships among various biological species or other entities based upon similarities and differences in their physical and/or genetic characteristics. The phylogenetic tree of beta-lactamase was constructed using Phylip 2.37 **[15].** One of the most commonly used tests for the reliability of phylogenetic tree is Felsenstein's (1985) **[15]** bootstrap test, which is evaluated using Efron's (1982) bootstrap resampling technique with replacement of informative sites thus in particular resampling results in some sites to be ignored while other taken into account **[31].** Surprisingly, with bootstrap 1000 we have obtained 100% bootstrap value for all internal nodes of all phylogenetic trees of beta-lactamases. This implies that phylogenetic trees constructed here are reliable.

The TEM-1 enzyme phylogenetic tree is clustered into four distinct subgroups. The phylogenetic tree of TEM-1 was rooted using ACJ43252 from *Escherichia Coli* as an outgroup **Figure S7 (Available with author).** The data indicate three occasions of gene duplications. Further, upon comparision of gene tree and species tree **Figure S8 & S7 (Available with author)** we have obtained incongruency in phylogenetic data. This implies occurrence of horizontal gene transfer where Escherichia *Coli* (ADL40232) gene was transferred to Pseudomonas Aeruginosa (ADL40245) **[32].**

Inspection of phylogenetic tree **Figure S9 (Available with author)** of class A enzyme including CTX-M-15 ant TOHO-1 suggest that AFN06802 variant from Escherichia Coli is an outgroup. One instance of HGT was noted in TOHO-1 where Escherechia Coli containing BAA07082 gene was transferred to Shiegella Flexneri (AAT76431). This implies TOHO-1 enzyme evolved via horizontal gene transfer which is also supported by recent studies that showed occurrence of HGT of TOHO-gene. **[33, 34].** The phylogenetic tree of SHV-1 enzyme **Figure S10 (Available with author)** shows that natural isolate AFN82169 from Klebsiella Pneumonia is an outgroup and gene duplication occurred in three instances. However, horizontal gene transfer was not observed in our analysis. Contrary to our findings, recent studies indicate that in 12 clonally distinct isolates of other variants of SHV-1, HGT occurred **[35].**

Phylogenetic tree analysis of classD beta-lactamases

The phylogenetic tree of OXA-10, a class D enzyme, reveals P14489 isolate from Pseudomonas Aeruginosa is an ancestor of all OXA-10 variants **Figure S11 (Available with author).** We noted two instances of HGT where uncultured bacterium (Q79R56) acquired the gene from Pseudomonas Aeruginosa (B6D9D4). In a similar vein, Pseudomonas Aeruginosa (B9W1Z5) acquired the gene from Providencia Stuartii (H6URT6). These observations are supported by occurrence of horizontal gene transfer in other variant of OXA-10 such as blaOXA-24/40 plasmid **[36].** Gene duplication event was not observed for OXA-10 enzyme. Thus, our data conclusively suggest that OXA-10 enzyme evolved by horizontal gene transfer mechanism.

Phylogenetic tree analysis of class B beta-lactamases

Examination of phylogenetic tree of VIM-2 **Figure S12 (Available with author)** demonstrate ACH43053 variant from *Pseudomonas Aeruginosa* is an outgroup. The phylogenetic data also indicate that *Achromobacter Xylosoxidans* containing AAT94165 gene was transferred to *Pseudomonas Pseudoalcaligenes* (AT90390). Recent studies have also reported the emergence of multidrug-resistant *Pseudomonas Putida* by horizontal transfer of VIM-2 resistance genes **[37].** Hence VIM2 evolved by HGT mechanism.

Inspection of phylogenetic tree of IMP **Figure S13 (Available with author)** indicate CAK55562 variant from *Pseudomonas Putida* is an outgroup. The phylogenetic data also display one case of gene duplication. However, HGT was not observed in IMP-1 phylogenetic tree. Conversely, horizontal gene transfer has been recently reported in IMP-1 gene **[38].**

Investigation of SIM-1 phylogenetic tree **Figure S14 (Available with author)** demonstrate that *Acinetobacter Baylyi* producing AER61546 variant is an outgroup and gene duplication occurred but horizontal gene transfer event was not observed. Contrary, to our findings HGT of SIM-1 resistance gene has been described **[39].** The phylogenetic tree of NDM-1 enzyme **Figure S15 (Available with author)** was rooted using variant AFP49131 from *Citobacter Freundii* as an out group. Three instances of gene duplication occurred. We did not observed horizontal gene transfer of NDM-1 gene. However, recent findings indicate occurrence of HGT of NDM-1 resistant gene among different bacterial species **[40].** These results imply that A, B and D classess of beta-lactamases evolved by different mechanisms.

Conclusion:

These studies have demonstrated structure-function relationship of three classes of beta-lactamases. The phylogenetic studies suggest that different classess of beta-lactamases evolved by different mechanisms.

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