Identification of Arg32Ser, His92Tyr and Leu147Phe novel mutations in chromosomally mediated β-lactamase SHV and in silico characterization to understand their substrate activity imparting resistance

Sangita Sarma,a,b Banani Dekaa,b, Pankaj Pradeep Panyangc and Anil Kumar Singha,b

aBiotechnology group, Biological Sciences and Technology Division, CSIR-North East Institute of Science and Technology, Jorhat, Assam, India; bAcademy of Scientific and Innovative Research (AcSIR), Ghaziabad, Uttar Pradesh, India; cDepartment of Pediatrics, Jorhat Medical College and Hospital, Jorhat, Assam, India

Communicated by Ramaswamy H. Sarma

ABSTRACT
The emergence of β-lactam resistance is yearning for clinical significance in Enterobacteriaceae, which are categorized under global priority pathogens list by the World Health Organization. Likewise, the prevalence of numerous β-lactamase enzymes, mutational propensity in such bacteria, and their role in accelerating resistance is still a major concern. Thus, the present work intends to characterize the β-lactamase producing bacteria isolated from acute diarrheal patients to understand their chromosomally acquired resistance pattern through molecular characterization and in silico approaches. The current study highlights the first identified Escherichia fergusonii and Escherichia marmotae species and their β-lactamase encoding genes, blaOKP-A, blaNDM and blaOXA from the unexplored Enterobacteriaceae family from North East India. First-ever reported point mutations such as Arg32Ser, His92Tyr, and Leu147Phe were observed in BlaSHV protein of two Klebsiella pneumoniae isolates S-35 and S-46. In molecular docking, non-catalytic site H-bond interactions of Arg 218, Ala 223, Asn 128, Ser 126, Gln 95, Asp 100, Tyr 101, Ser 102, Ala 274 with a low binding affinity towards BlaSHV was found. This correlates with the high imipenem, ceftazidime, cefuroxime, ceftriaxone, and cefpodoxime resistance in Klebsiella pneumoniae S-35 with the complementary effect of mutations Arg32Ser and Leu147Phe. Besides, the role of His92Tyr mutation in controlling the resistance in Klebsiella pneumoniae S-46 is also illustrated. Thus, our study highlights the novel mutations of β-lactamase and its clinical importance with altered resistance profiles. This could be useful to design better therapeutics and to readjust antibiotic treatment regimes against them and control to grow more resistance under selective pressure.

Introduction
The rapid incidence of global antibiotic resistance has been yielded by numerous resistance-causing pathogens in the recent past. In this aspect, World Health Organization (WHO) has categorized such resistance carrying pathogens into three major groups critical, high, and medium against which new therapeutics development needs in high priority (Datta et al., 2012; Mulani et al., 2019; World Health Organization, 2019). Among the WHO critical group, third-generation cephalosporins and carbapenem-resistant Enterobacteriaceae bacteria desire generous attention concerning their crucial role in emerging resistance (World Health Organization, 2019). Besides, characterization of this Enterobacteriaceae family pathogen is very much essential and various virulence indulging factors and genomic diversity could analyze their disease transmission rate over multiple generations. The β-lactam antibiotics, especially carbapenems and cephalosporins, are widely used without much consideration to resistance caused by the development of the β-lactamase enzyme (Dallenne et al., 2010). Chromosomal β-lactamases can be regarded as constitutive due to the presence of some mutations in the bacterial genome (Garau, 1994). Through the evolution of various classes of the β-lactamase enzyme, the antibiotics substrate specificities also differ relating to several point mutations (Bush & Fisher, 2011). Moreover, the catalytic and non-catalytic site amino acid residues also contribute to the wider spectrum activity of an antibiotic (Palzkill, 2018).

With the abundance of sources like environmental or non-pathogenic origin, class A CTX-M type broad-spectrum and extended-spectrum β-lactamase (ESBL) group has covered a maximum space with their intensifying antibiotic resistance, and out of them, group 1 enzyme blaCTX-M-15 was found as predominant (Livermore & Hawkey, 2005). To date, >100 variants of the class A group of ESBL SHV enzymes have been found worldwide, based on their evolutionary changes through mutations (Bradford, 2001; Ling et al., 2012; Mulani et al., 2019; Dallenne et al., 2010). The emergence of β-lactam resistance is yearning for clinical significance in Enterobacteriaceae, which are categorized under global priority pathogens list by the World Health Organization. Likewise, the prevalence of numerous β-lactamase enzymes, mutational propensity in such bacteria, and their role in accelerating resistance is still a major concern. Thus, the present work intends to characterize the β-lactamase producing bacteria isolated from acute diarrheal patients to understand their chromosomally acquired resistance pattern through molecular characterization and in silico approaches. The current study highlights the first identified Escherichia fergusonii and Escherichia marmotae species and their β-lactamase encoding genes, blaOKP-A, blaNDM and blaOXA from the unexplored Enterobacteriaceae family from North East India. First-ever reported point mutations such as Arg32Ser, His92Tyr, and Leu147Phe were observed in BlaSHV protein of two Klebsiella pneumoniae isolates S-35 and S-46. In molecular docking, non-catalytic site H-bond interactions of Arg 218, Ala 223, Asn 128, Ser 126, Gln 95, Asp 100, Tyr 101, Ser 102, Ala 274 with a low binding affinity towards BlaSHV was found. This correlates with the high imipenem, ceftazidime, cefuroxime, ceftriaxone, and cefpodoxime resistance in Klebsiella pneumoniae S-35 with the complementary effect of mutations Arg32Ser and Leu147Phe. Besides, the role of His92Tyr mutation in controlling the resistance in Klebsiella pneumoniae S-46 is also illustrated. Thus, our study highlights the novel mutations of β-lactamase and its clinical importance with altered resistance profiles. This could be useful to design better therapeutics and to readjust antibiotic treatment regimes against them and control to grow more resistance under selective pressure.

CONTACT Anil Kumar Singh 1010anil@gmail.com Biotechnology Group, Biological Sciences, and Technology Division, CSIR-North East Institute of Science and Technology, Jorhat, Assam, India

Supplemental data for this article can be accessed online at https://doi.org/10.1080/07391102.2021.1978321.

This article has been republished with minor changes. These changes do not impact the academic content of the article.
Among the innumerable number of genes in four major \( \beta \)-lactamase classes, the class B group of \( \text{blaNDM} \) carries paramount importance over other \( \beta \)-lactamase classes, with its horizontal gene transferability and hyper resistance activity towards most of all \( \beta \)-lactam antibiotics (Bennett, 2008; Mishra et al., 2013). Since the year 1989, the entire world is experiencing plasmid-mediated resistance caused by AmpC genes with the gradual increment of the class C group of AmpC \( \beta \)-lactamase production (Niumsup et al., 2003; Walther-Rasmussen & Heiby, 2002). Another prior discovered \( \beta \)-lactamases is a class D group of oxacillin hydrolyzing OXA enzyme, which causes plasmid and chromosomal mediated resistance along with its numerous variants (Evans & Amyes, 2014).

From North-East India, no studies are reported on molecular characterization of \( \beta \)-lactamase aided gene mutation concerning the emergence of \( \beta \)-lactam resistance. To unveil the importance of various groups of enteric bacteria during the drug-resistance outbreak, the present study highlights some new chromosomal mediated \( \beta \)-lactamase encoding genes like \( \text{blaOXA} \) and \( \text{blaNDM} \) prevalence in several unexplored \textit{Enterobacteriaceae} bacterial species like \textit{Escherichia fergusonii} and \textit{Escherichia marmotae} collected from acute diarrheal patients. Besides, the \( \text{blaOKP-A} \) gene is reported for the first time in \textit{K. pneumoniae} species from this region. We have also enquired about the mutational diversity among the chromosomal enzymes and our results demonstrate that non-cata-lytic site point mutation such as Arg32Ser, His92Tyr, and Leu147Phe in the BlaSHV enzyme is solely responsible for mounting resistance in imipenem, ceftazidime, cefuroxime, ceftriaxone, and cefpodoxime antibiotics. In addition to mutation, a group of amino acid residues involved in H-bond interaction between BlaSHV enzyme and antibiotics substrate with lower binding affinity also contributes to the higher resistance pattern of \textit{K. pneumoniae} isolates. As a result, our study could abridge the therapeutics problem corresponds to broad-spectrum carbapenem and cephalosporin antibiotics through \textit{in silico} and molecular characterization study of the \( \beta \)-lactamase enzyme relating various \( \beta \)-lactam substrates.

Materials and methods

\textbf{The details of bacterial isolates and antibiotics used in the study}

This study was conducted with the bacterial samples collection between 2015-2016. A total of 29 clinical isolates were collected from acute diarrheal children of age group 1.5-11 years from the Paediatrics department of Jorhat Medical College and Hospital, Assam, India to perform the various phenotypic, genotypic and \textit{in silico} studies. The nine \( \beta \)-lactam antibiotics viz. ertapenem sodium salt, doripenem hydrate, meropenem trihydrate, imipenem, cefuroxime axetil, ceftriaxone disodium salt hemi (heptahydrate), cefoxitin sodium salt, cefpodoxime and ceftazidime pentahydrate were purchased from Sigma Aldrich, USA and stocks were prepared following standard protocol.

\textbf{16S rRNA sequencing for bacterial identification and phylogenetic assessment}

To identify 29 clinical isolates, 16S rRNA sequencing was performed using 16S rRNA universal primers (Supplementary Table S1). In brief, genomic DNA for all the isolates was extracted using the Lysozyme method (Medjahed & Singh, 2010) and DNA concentration was measured using Nanodrop™ 1000 spectrophotometer (Eppendorf). Approximately 1500 bp 16S rRNA sequences of 29 isolates were edited by Bioedit v7.0.5 for submission in NCBI/GeneBank server and accession numbers were collected. 235 rRNA sequence (3038 bp length) of \textit{Acidianus hospitalis} strain W1 (NR_103012.1) was served as an outgroup in determining rooted tree. Additionally, the rooted phylogenetic tree was constructed for all the twenty nine 16S rRNA sequences of clinical isolates of ~1 Kb length through the Maximum likelihood and Tamura-Nei model, based on 1000 bootstrap replicates in MEGAX software (Ciccozzi et al., 2019; Kumar et al., 2018; Tamura & Nei, 1993).

\textbf{\( \beta \)-lactamase activity determination}

To check the individual \( \beta \)-lactamase activity (\( \beta \)-L Activity) of all the 29 isolates, a colourimetric assay was performed using a commercial kit from Biovision (Milpitas, CA, USA) with a little modification in the protocol (Jeon et al., 2017). In brief, 25 \( \mu \)L of total samples were added to a 96-well cell flat-bottomed culture plate followed by the addition of nitrocefin to the 20 \( \mu \)M final concentration. Subsequently, absorbance at 490 nm was measured using a Synergy 2 multi-mode plate reader (BioTek, Winooski, VT, USA) for 30 min, keeping 10 min intervals and nitrocefin hydrolysis curve was obtained. A nitrocefin standard curve was plotted and sample \( \beta \)-L Activity was determined using manufacturers protocol which can be expressed as mU/mg of protein.

\textbf{Antibiotics susceptibility test}

An antibiotic susceptibility test was conducted with nine \( \beta \)-lactam antibiotics using a previously described method (Stavri et al., 2004). The incubation period of 16-24 h at 37°C was maintained and the bacterial growth confirmation was done by adding 10 mg/mL MTT (HiMedia, India) followed by 20 min incubation at 37°C. The experiment was carried out in triplicates with suitable controls (DMSO, bacterial cell and growth media).

\textbf{Phenotypic test for the detection of \( \beta \)-lactamase production}

\textbf{Carbapenemase detection test}

Prevalence of carbapenemase enzyme was confirmed by Carba NP test, class A KPC and class B MBL (VIM, IMP and NDM etc) detection test. For KPC and MBL enzyme conformation, ertapenem with & without boronic acid Ezy MIC Strips, meropenem with & without EDTA Ezy MIC Strips and imipenem with & without EDTA Ezy MIC Strips obtained from Himedia, India were used. CLSI guidelines were applied in analyzing the enzyme occurrence among 29 clinical isolates.
Carba NP test was performed to detect carbapenemase-producing ability among the clinical isolates with a little modification of the previous method using 2 hr incubation period (Pasteran et al., 2015). E. coli ATCC 25977 was used as a negative control.

**ESBL detection test**

For class A ESBL enzymes TEM, CTX-M and SHV detection, Ceftazidime/Ceftazidime + Clavulanic acid Ezy MIC Strip and Ceftriaxone/Ceftriaxone + Clavulanic acid Ezy MIC Strip kit were procured from Himedia, India. CLSI guidelines were procured from Himedia, India. CLSI guidelines were applied according to the manufacturer’s protocol for the identification of ESBL enzymes in 29 clinical isolates.

**Genotypic detection of β-lactamase producing isolates**

All 29 clinical isolates were utilized for the genotypic test to examine various ESBL and carbapenemase genes present in their chromosome. For this study, PCR amplification was carried out with various sets of gene-specific primers shown in Table S1. The polymerase chain reaction (PCR) was carried out for a 25 μl reaction mixture containing genomic DNA as a template. The thermal cycle set up was found as an initial step of denaturation at 95°C for 5 min, 35 cycles of DNA denaturation for 30 s, annealing of primers at 41°C (blaTEM), 47°C (blaNDM, blaKPC-1), 53°C (blaOKP), 48°C (blaMIR), 50°C (blaCTX-M), 51°C (blaIMP), 53°C (blaSHV), 52°C (blaMOX, blaCMY-1, blaOXA-48, blaVIM) and 59°C (blaOXA-1) for the 30 s, extension step at 72°C for 10 min followed by a final extension at 4°C for the ∞ period. Sanger sequencing was carried out for the respective PCR products and accession numbers were generated by submitting all the gene sequences in NCBI/BankIt server.

**In silico analysis of β-lactamase protein**

**Molecular modelling of SHV protein**

Due to the unavailability of the 3-dimensional (3D) crystallographic PDB structure of the wild type (WT) and mutant BlaSHV protein of the four *Klebsiella pneumoniae* isolates, we have performed homology-based modelling to predict their 3D structures. The translated amino acid sequences of the blaSHV gene were initially deposited in the Swiss-Model protein homology prediction server (Waterhouse et al., 2018) to conduct reference-based 3D PDB structure prediction. Further, based on ~100% sequence homology with BlaSHV-11 from *Klebsiella pneumoniae* strains, matchmaker structure comparison of two modelled mutant SHV protein with wild type SHV-11 protein (PDB ID: 6NF D) was carried out using Chimera 1.15 software to gather information on the secondary structural diversity in superimposed structures.

**Prediction of SHV model protein secondary structure**

For characterization of secondary structure, PSI-blast-based secondary structure prediction (PSIPRED) (Buchan & Jones, 2019; Jones, 1999), self-optimized prediction method with alignment (SOPMA) (Geourjon & Deleage, 1995) and ExPasy ProtParam tools online server (Gasteiger et al., 2005) were utilized in BlaSHV model protein.

**Binding site prediction of SHV protein with the β-lactam substrate through molecular docking**

The 3D structures of Carbapenem and cephalosporin drug meropenem, ertapenem, doripenem, imipenem, ceftazidime, cefotixin, ceftriaxone, cefuroxime, and cefpodoxime were retrieved from PubChem compound database (https://pubchem.ncbi.nlm.nih.gov/compound/) in SDF format and converted to PDB format using Open Babel 3.1.1 software (https://pypi.org/project/openbabel/3.1.1/). Molecular docking was performed using AutoDock tools 1.5.6. Using a 60 × 60 × 60 Å grid box in the WT and mutant BlaSHV protein, an initial Autogrid program was run to find catalytic and non-catalytic binding site residues, followed by an Autodock program based on the Lamarckian Genetic Algorithm (Dhara et al., 2013). The protein-substrate complexes with maximum binding energy were visualized in Chimera 1.15 software to get binding site amino acid residues and hydrogen bonding interaction information.

**Results**

**Bacterial identification and phylogenetic relationship of clinical isolates**

Among 29 acute diarrhoeal isolates, five different categories of *Enterobacteriaceae* family bacteria were found as *Enterococcus* sp. (6.89%), *Klebsiella* sp. (31.03%), *Escherichia* sp. (44.82%), *Shigella* sp. (13.79%) and *Acinetobacter* sp. (3.44%) as shown in Table S2. A higher abundance of *Escherichia* sp. isolates were found and among them, the very first time we could report 9 *Escherichia fergusonii* and 2 *Escherichia marmota* strains from North East India.

After bacterial identification, their evolutionary relationship was inferred by the maximum-likelihood cladoistics distance matrix method. In this analysis, the phylogenetic tree was found to be subdivided into three clusters viz. clusters I, II and III. The circular tree comprising of three clusters represents the chronological order of evolutionary relatedness of 29 isolates as shown in Figure 1. Cluster I tends to be a large clade comprising multiple small sub-clusters. The arrangements of Cluster I subclusters were found to be distributed as (i) Monophyletic association of *Klebsiella pneumoniae* S-2 & *Klebsiella pneumoniae* S-35 with the paraphyletic clade of *Klebsiella pneumoniae* S-13, (ii) Monophyletic association of *Shigella dysenteriae* S-39 & *Escherichia* sp. S-48 and *Shigella flexneri* S-38 in paraphyletic relationship with *K. pneumoniae* S-2, *K. pneumoniae* S-35, *K. pneumoniae* S-13, *Shigella dysenteriae* S-39 and *Escherichia* sp. S-48, (iii) Monophyletic association of *Shigella sonnei* S-33 & *Shigella flexneri* S-42, *E. fergusonii* S-10 & *E. fergusonii* S-14 in a paraphyletic association with *E. fergusonii* S-3, (iv) Monophyletic association of *Escherichia* sp. S-6 & *E. fergusonii* S-32 was found to be related in a paraphyletic manner with *E. fergusonii* S-7 and (v) *K. pneumoniae* S-5 & *K. pneumoniae* S-16 shares a monophyletic relationship and connected in a paraphyletic clade of *Enterococcus faecium* S-1 and *Acinetobacter pittii* S-4. Similarly, Cluster II can also be subdivided into the following order: (i) Monophyly was observed between *K. quasipneumoniae* S-36 & *K. pneumoniae* S-46, (ii) The paraphyletic
relationship was found among *E. marmotae* S-11, *E. marmotae* S-52 with *K. quasipneumoniae* S-36 & *K. pneumoniae* S-46 and (iii) S-19 was found to be associated in a paraphyletic manner with S-36, S-46, S-11 and S-52. Lastly, in cluster III, the phylogenetic associations were observed as: (i) *K. pneumoniae* S-9 and *K. pneumoniae* S-45 resides in a monophyletic clade and (ii) *E. fergusonii* S-8, *E. fergusonii* S-30 and *E. fergusonii* S-53 in a paraphyletic relationship with *K. pneumoniae* S-9 and *K. pneumoniae* S-45.

**High β-lactam resistance among clinical isolates**

The incidence of multidrug-resistant (MDR) *Enterobacteriaceae* strain emerging globally (Savard & Perl, 2012). Therefore, in this present study, we have investigated the β-lactam resistance profile of 29 clinical isolates to understand their role in disease transmission. β-lactam antibiotics class especially broad-spectrum carbapenem and extended-spectrum second and third-generation cephalosporin was used for determining their minimum inhibitory concentration (MIC). The antibiotic susceptibility test revealed high extended-spectrum propensity among three isolates *K. pneumoniae* S-2, *E. fergusonii* S-10 and *S. dysenteriae* S-39 amongst 29 isolates by showing high MIC. For all the 29 isolates, MIC values are listed in Table 1. S-2, S-10 and S-39 exhibited high to moderate range of MIC for all four carbapenem antibiotics imipenem (>200, >400 and 100 μg/ml), meropenem (50, 25 and 12.5 μg/ml), doripenem (200, 6.25 and 0.78 μg/ml) and ertapenem (200, >400 and 3.12 μg/ml). Similarly, second-generation cephalosporin cefoxitin (800, 400 and 200 μg/ml) and cefuroxime (>200, 200 and 800 μg/ml) exhibited higher resistance. Third-generation cephalosporin ceftazidime (100, 800 and 100 μg/ml), ceftriaxone (>1600, >1600

---

**Figure 1.** Maximum likelihood phylogenetic relationship study of 29 clinical isolates using MEGA X.
Table 1. Antibiotic susceptibility profile of carbapenem and cephalosporin group of antibiotics against 29 clinical isolates.

| Antibiotics | MIC (µg/ml) | Acinetobacter sp. | Escherichia sp. | Enterococcus sp. | Klebsiella sp. | Shigella sp. |
|-------------|------------|-------------------|-----------------|-----------------|----------------|-------------|
| Imipenem    | <0.09      | >1600              | 200              | >1600            | >1600          | >1600       |
| Meropenem   | <0.09      | >1600              | 200              | >1600            | >1600          | >1600       |
| Ertapenem   | <0.09      | >1600              | 200              | >1600            | >1600          | >1600       |
| Doripenem   | <0.09      | >1600              | 200              | >1600            | >1600          | >1600       |
| Ceftazidime | <0.09      | >1600              | 200              | >1600            | >1600          | >1600       |
| Cefoxitin   | 0.39       | >1600              | 200              | >1600            | >1600          | >1600       |
| Cefuroxime  | 1.56       | >1600              | 200              | >1600            | >1600          | >1600       |
| Cefpodoxime | 3.12       | >1600              | 200              | >1600            | >1600          | >1600       |

The phenotypic and genotypic test revealed high ESBL and low carbapenemase producers among the clinical isolates

Once the higher resistance tendency has been revealed among the various group of clinical isolates, the phenotypic test was performed to understand their enzymatic activity imparting resistance. In this section, the colourimetric assay of ß-lactamase activity and two major ß-lactamase classes like ESBL and carbapenemase activity was determined for 29 clinical isolates. In the colourimetric assay, the highest activity in 490 nm absorbance was exerted by four isolates S-10 (20.22 mU/mg) from Escherichia species, S-16 (26.65 μg/mL) and S-2 (20.72 μg/mL) from Klebsiella species, and S-39 (18.22 μg/mL) from Shigella species. Compared to a positive control (23.64 μg/mL), all these three isolates were found to share approximately similar ß-lactamase activity except S-16 with higher activity than positive control as shown in Figure 3. On the other hand, S-4 from Acinetobacter species (5.22 μg/mL), S-19 from Enterococcus species (5.32 μg/mL), S-11 from Escherichia species (4.91 μg/mL), S-45 from Klebsiella species (4.23 μg/mL), and S-33 from Shigella species (5.36 μg/mL) exerts the lowest activity.

The colourimetric assay detected the overall ß-lactamase activity of all the 29 isolates without distinguishing them in any specific class. Henceforth, in this part, we have tried to examine their specific enzymatic activity by performing ESBL and carbapenemase (MBL, K. pneumoniae carbapenemase KPC, OXA) detection tests. Through the carbapenemase detection test carba NP, (Figure S1), clinical isolates S-7, S-8, S-10, S-11, S-30, S-39, S-45, S-48 and S-52 were found positive for carbapenemase production. Another two carbapenemase KPC and MBL enzyme detection were performed to observe any changes among the three carbapenemase tests. Through the MBL test (Meropenem with & without EDTA Ezy MIC Strips (Figure S4) and Imipenem with & without EDTA Ezy MIC Strips (Figure S5)) S-2, S-3, S-4, S-10, S-16, S-29, S-38, S-53 and S-52, two sets of isolates were found to be positive and KPC test (Ertapenem/Ertapenem + Boronic acid Ezy MIC Strips (Figure S6) had identified S-10 and S-45 as positive isolates. Again, ESBL test (Ceftazidime/Ceftazidime + Clavulanic acid Ezy MIC Strips (Figure S2) and Ceftriaxone/Ceftriaxone + Clavulanic acid Ezy MIC Strips (Figure S3) could divulge a set of second and third-generation cephalosporin-resistant isolates like S-5, S-9, S-11, S-13, S-14, S-29, S-30, S-35, S-39, S-52 and S-5, S-9, S-11, S-13, S-29, S-30, S-32, S-35, S-39, S-52 as ESBL positive strains respectively considering Ceftazidime/Ceftazidime + Clavulanic acid zone diameter and >1600 μg/mL) and cefpodoxime (>200, >200 and 12.5 µg/ml) also showed higher resistance pattern. The heatmap (Figure 2) represents the variation in MIC pattern of each isolates in a graphical representation showing MIC range from <0.09 - >1600 μg/mL. Among 29 isolates, four carbapenem antibiotics, i.e. imipenem, meropenem, ertapenem and doripenem have shown 93.1%, 44.82%, 31.03% and 0.1% resistance respectively and for five cephalosporins, i.e. cefoxitin, cefuroxime, cefazidime, ceftriaxone and cefpodoxime resistance was 34.48%, 75.86%, 93.1%, 79.31% and 58.62% respectively.
as >8 mm following CLSI guidelines mentioned in manufacturer’s instruction.

After gathering the phenotypic test knowledge, the existence of enzyme was further clarified by conducting genotypic tests by taking twelve β-lactamase encoding genes corresponding to four β-lactamase classes A, B, C and D into consideration. The PCR results revealed 22 various β-lactamase genes among different groups of isolates and their accession numbers are listed in Table S3. Class A carbapenemase KPC was not present in any of the isolates. On the other hand, class A ESBL enzyme blaTEM was found in E. fergusonii isolates S-10 and S-30 and E. marmotae S-52. All three blaTEM genes in this study shared 100% similarities with the complete sequence of blaTEM gene of K. pneumoniae LYS 105A plasmid pLYS105A-2 isolated from the liver of peafowl in China. blaSHV is one more ESBL enzyme from the class A group and prevalent in E. marmotae isolates S-9, S-13, S-35 and S-46. Through NCBI BLAST analysis, the blaSHV gene sequence of S-9 shared 100% homology with a blaSHV-28 variant of K. pneumoniae E16KP0102 complete genome sequence from the human blood sample in South Korea. blaSHV partial CDS of S-13 covered 100% homology with K. pneumoniae blaSHV-63 complete CDS from the USA. Another two blaSHV genes from S-35 and S-46 in this study displayed 100% and 99% sequence similarity with blaSHV-11 variants of K. pneumoniae KP20194a complete genome and K. pneumoniae C16KP0108 plasmid pC16KP0108-2 complete genome sequence from human sputum and blood sample in China and South Korea respectively. Another class A group of non-ESBL and limited-spectrum β-lactamases (LSBLs) (Philippou et al., 2016) encoding gene blaOXA was identified only in K. pneumoniae S-2. Class A enzyme blaOXA-A was found to share 99.76% similarity to the allelic variant blaOXA-A-11 gene product of K. quasipneumoniae subsp. quasipneumoniae strain M17277 chromosome. One of the most predominant variants of class A ESBL is blaCTX-M-15 (Yu et al., 2007) and were found in E. fergusonii S-8. On the other hand, blaCTX-M has extensively occurred in E. fergusonii isolates S-10, S-14, S-29 and S-30, E. marmotae isolates S-11, and S-52, K. pneumoniae S-9, and S. dysenteriae S-39. All the nine allelic variants shared 100% similarity with the complete sequence of K. pneumoniae subsp. pneumoniae strain 27B plasmid pKpQIL, except S-10 with 99% similarity.

Class B group of carbapenemase MBL encoding gene blaNDM was present in E. fergusonii S-8 and E. fergusonii S-10, whereas other two MBL genes blaIMP and blaVIM was not found in any of the isolates.

Some other β-lactamase genes like the class C group of AmpC encoding genes blaMOX, blaMIR and blaCMY were also studied and none of the isolates was shown their existence in the chromosome.

Class D group of oxacillin hydrolyzing carbapenemase blaOXA was recognized in E. fergusonii S-10, E. marmotae S-11 and S. dysenteriae S-39. The partial CDS sequence of blaOXA was found to share 100% similarity with the complete sequence of the blaOXA-1 product of K. pneumoniae subsp. pneumoniae strain 27B plasmid pKpQIL. While another class D enzyme blaOXA-48 was absent in all the 29 isolates. Figure S7-S11 include all the results of four β-lactamase classes.

**In silico characterization of β-lactamase encoding proteins**

From the genotypic characterization of four β-lactamase classes, the nucleotide homology of the 22 genes were retrieved and in this section, we have tried to investigate chromosomal mutation in them. For this, 22 translated protein sequences of the respective β-lactamase encoded genes were aligned with publicly available reference protein sequences (Supplementary Figure S12-S17). In the current findings, Class A ESBL BlaCTX-M of E. fergusonii S-10 strain was marked with a silent mutation of Val288Val (TCC→TC846G) while comparing with other three BlaCTX-M-15 proteins (QTG66273.1, M4TKM6, 4HBT_1). Two other class A ESBL BlaSHV proteins from K. pneumoniae S-35 and K. pneumoniae S-46 were detected with Arg32Ser and Leu147Phe, and Arg32Ser, His92Tyr, and Leu147Phe mutation respectively while comparing with the BlaSHV-11 protein sequence of K. pneumoniae (QKT87143.1, A0A291FB61, 6NFD_1) (Figure 4). On the other hand, class A ESBL BlaTEM of S-10, S-
30, S-52, BlaSHV of S-9, S-13, ESBL BlaCTX-M of S-8, S-9, S-14, S-29, S-30, S-39, S-52 and non-ESBL BlaOKP-A of S-2 have shown no mutations. Additionally, class B and class D carbapenemase BlaNDM of S-8, S-10 and BlaOXA of S-10, S-11, S-39 also exhibited no mutations in their protein alignment (Figure S12-S17).

Once the mutations were recorded for two BlaSHV proteins from *K. pneumoniae* strain S-35 and S-46, their 3D structures were studied by taking two control BlaSHV proteins of *K. pneumoniae* strain S-9 and S-13 carrying no mutation. All the 3D models generated from the Swiss-Model server were validated for their structural assessment as shown in Figure S18 and S19. Through the MolProbity program, 1.08 Mol Probity score, 0.49 clash score, 0.39% Ramachandran outliers, rotamer outliers 1.94%, 96.90% Ramachandran favoured region were predicted for S-9 BlaSHV protein. For S-13 BlaSHV protein, 1.07% Mol Probity score, 0.49% clash score, 0.77% Ramachandran outliers, rotamer outliers 1.93%, 96.91% Ramachandran favoured region were detected. For S-35 BlaSHV protein, 0.82% Mol Probity score, 0.00% clash score, 0.40% Ramachandran outliers, rotamer outliers 1.48%, 96.83% Ramachandran favoured region were obtained. Similarly, S-46 BlaSHV analysis revealed 0.98 Mol Probity score, 0.49 clash score, 0.39% Ramachandran outliers, 1.46% rotamer outliers and 96.90% Ramachandran favoured region. Quality assessment was done by evaluating QMEAN Z-score for each of the modelled proteins comparing with the template ESBL Arg164His mutant BlaSHV-1 (PDB ID: 3opl.1.A) as shown in Figure S18 & S19. Form S-9, S-13, S-35

**Figure 3.** β-lactamase activity of 29 isolates in 10 μl culture with respect to positive control (*E. coli*) and negative control (DMSO) per mg of protein namely: (a) *Acinetobacter* species (b) *Enterococcus* species, (c) *Escherichia* species, (d) *Klebsiella* species and (e) *Shigella* species.
and S-46 BlaSHV protein, in the global quality assessment, QMEAN Z-score was found as 0.10, 0.11, 0.28 and 0.16 indicating the Cβ atom interaction capacity (Santhosh Kumar & Yusuf, 2020). Also, the comparison plot between the
template and modelled structure denotes the quality score of the model’s protein size which can be related to template size (Figure S18 & S19).

In terms of secondary structure prediction for all the control as well as mutant modelled protein structures, the SOPMA analysis divulges that S-9, S-13, S-35 and S-46 BlaSHV protein contains, alpha helix, extended beta-sheets, beta-turn and random coils among which (alpha-helix: correspondingly 45.55%, 47.87%, 45.09% and 46%) and (random coils: 33.45%, 32.62%, 33.82% and 32.74%) were predominantly found (Table S4). Similarly, ExPasy ProtParam tool analysis reveals amino acid composition in the modelled protein structures and found that alanine (Ala) and leucine (Leu) percentage was comparatively higher (12.8%, 12.8%, 12.4% and 12.8%), (12.5%, 12.8%, 12.4%, and 12.5%) respectively as shown in Table S5. PSIPRED analysis also classified various categories of amino acids as small non-polar, hydrophobic, polar and aromatic plus cysteine as shown in Figure S20 & S21.

After analyzing the modelled protein structures, the enzyme-substrate activity were measured in order to infer their role in resistance. For determining the binding activity of beta-lactam substrates with control and mutant BlaSHV enzyme, molecular docking approach was adopted. For enzyme + substrates binding, we observed ten conformations each for nine docking complexes of broad spectrum carbapenem doripenem, ertapenem, imipenem and meropenem binding site with corresponding amino acid residues.

Among S-9+ carbapenem substrate complexes, doripenem exhibited the highest binding energy (-7.89 Kcal/mol) and imipenem showed the lowest (-5.34 Kcal/mol). For S-9+ cephalosporin substrate complexes, ceftazidime (8.0 Kcal/mol) and ceftazidime (-5.85 Kcal/mol) were represented with the highest and lowest energy (Figure 5). Also, for S-13+ carbapenem substrate complexes, meropenem (-7.62 Kcal/mol) and imipenem (-4.19 Kcal/mol) contributed to the highest and lowest binding energy, while for S-13+ cephalosporin substrate, ceftriaxone (-7.88 Kcal/mol) and ceftazidime (-6.49 Kcal/mol) contributed to the highest and lowest binding energy (Figure 6). Similarly, for S-35+ carbapenem complexes, doripenem (-6.49 Kcal/mol) and ertapenem (-4.42 Kcal/mol) have corresponded to highest and lowest energy showing substrates and cefoxitin (-7.41 Kcal/mol) and cefuroxime (-5.22 Kcal/mol) for S-35+ cephalosporin complexes. On the other hand, for S-46+ carbapenem complexes, ertapenem (-6.64 Kcal/mol) and imipenem (-4.59 Kcal/mol) stand for the highest and lowest binding affinity and S-46+ carbapenem complexes, ceftriaxone (-6.65 Kcal/mol) and ceftazidime (-4.96 Kcal/mol). The binding pocket along with neighbouring amino acid residues of each substrate abundantly showed hydrophobic contacts along with all the H-bonding and other binding site residues for 22 enzyme + substrate complexes (Table 2).
Table 2. Molecular docking results of BlaSHV protein of WT and mutant strain S-9, S-13 and S-35, S-46 respectively with corresponding details of binding energy and interactive amino acid residues.

| β-lactam Substrates | H-bonding residue | Other binding site residue | Binding energy |
|----------------------|-------------------|---------------------------|---------------|
| **Cefazidime**       |                   |                           |               |
| MW: 546.6 g/mol      |                   |                           |               |
| Thr 114, Gln 202, Arg 211 | Trp 225, Asn 249 | Asp100, Ser126, Asn128 |               |
| Ser 66, Ser 126, Thr 163, Ala 233 | Ile227, Arg128, Glu 116, Ile 123, Thr 124, Met 125, Trp 206, Val 208, Pro 222, Ala 223, Gly 224, Arg 218, Phe 226, Ile 227 | Gly 205, Val 208, Pro 222, Ala 223, Gly 224, Arg 218, Phe 226, Ile 227 | Tyr101, Val212, Ala123, Lys230, Thr231, Gly232, Ala233 | Ile217, Ala223, Gly224, Trp225, Phe226, Ala228 | –5.85 | –6.49 | –5.94 | –4.96 |
| **Cefpodoxime**      |                   |                           |               |
| MW: 427.5 g/mol      |                   |                           |               |
| Arg 218, Gly 224, Trp 225, Phe 226, Ala 223, Asn 249 | Ser 66, Ser 126, Thr 163, Ala 233 | – | Ser 66, Ser 126, Thr 163, Ala 233 | Ser 66, Tyr 101, Ser 126, Asn 128, Asn 249, Val 212, Lys 230, Gly 232, Glu 235, Gly 234, Ile 227, Asp 210 | Gly 164, Asn 166, Lys 230, Gly 232, Glu 235, Gly 234, Ile 227, Asp 210 | Thl163, Leu165, Ala233, Gly234 | –7.09 | –7.88 | –6.82 | –6.37 |
| **Cefoxitin**        |                   |                           |               |
| MW: 427.5 g/mol      |                   |                           |               |
| Ser 219, Gly 224, Trp 225, Asn 249 | Ala233 | Ala274 | Ser126 | Arg 218, Val 220, Leu 221, Pro 222, Ala 223, Phe 226, Ile 227 | Ser 66, Tyr 101, Ser 126, Asn 128, Asn 249, Val 212, Lys 230, Ala 233 | Thr163, Leu165, Ala233, Gly234 | –7.44 | –7.01 | –7.41 | –5.57 |
| **Ceftriaxone**      |                   |                           |               |
| MW: 554.6 g/mol      |                   |                           |               |
| Ser 66, Asp 100, Ser 126, Thr 163, Ala 166, Ala 233 | Arg 198, Arg 218, Ile 227 | Ala274 | Ala223, Trp225, Phe226, Asn249 | Met 65, Tyr 101, Glu 164, Ala 165, Gly 232, Gly 234 | Arg 201, Gln 202, Leu 204, Val 205, Gly 208, Phe 226 | Val208, Leu221, Ala223, Gly224, Leu245, Val255, Val256, Ile273, Ala274, Gly275 | –8.0 | –7.02 | –5.33 | –6.65 |
| **Cefuroxime**       |                   |                           |               |
| MW: 424.4 g/mol      |                   |                           |               |
| Gln 205, Ala 251, Asn 249 | Gln205, Ala249 | Gln95, Tyr101, Ser102 | Arg194 | Val 208, Arg 218, Pro 222, Ala 223, Ile 227, Phe 226 | Leu98, Val99, Tyr101, His108 | Ala78, Val80, Ala82, Leu195 | –7.81 | –6.54 | –5.22 | –5.07 |
| **Doripenem**        |                   |                           |               |
| MW: 420.5 g/mol      |                   |                           |               |
| Trp 225, Ile 227, Ser 66, Asn 128, Ser66, Asn128 | Gln202, Trp206 | Gln202, Trp206 | Lys 69, Tyr 101, Asp 125, Ser 126, Ala 233, Gly 234, Glu 235 | Tyr101, Val212, Ala213, Lys230, Thr231, Ala233, Gly234 | Leu87, Gly116, Ala120, Leu121, Val203, Trp206 | –7.89 | –6.7 | –4.42 | –4.79 |
| **Ertapenem**        |                   |                           |               |
| MW: 497.5 g/mol      |                   |                           |               |
| Thr 137, Gly 139, Glu 235, Asn 270 | Arg218, Asn249 | Val138, Leu195 | Ser 66, Ser 126, Asn 166, Val 212, Ala 223, Gly 233, Gly 234, Arg 239 | Val208, Val220, Ala221, Ala223, Phe226, Ile227, Ala228 | Ala75, Val76, Ala78, Tyr101, Val138, Gly139, Gly140, Val143, Val144, Thr145, Ala146, Phe147, Leu195 | –6.02 | –6.78 | –6.49 | –6.64 |
| **Imipenem**         |                   |                           |               |
| MW: 299.35 g/mol     |                   |                           |               |
| Ser 66, Ser 126, Arg218, Ala223 | Asp127 | Met 65, Tyr 101, Ser 126, Asp 127, Val 212, Lys 230, Thr 231, Gln 232, Ala 233 | Met 65, Lys 69, Tyr 101, Val 212, Thr 231, Gly 232, Ala 233 | Met207, Val208, Val220, Ala221, Ala223, Gly224, Trp225, Phe226, Ala228 | –5.34 | –4.19 | –5.25 | –4.59 |

(continued)
Discussions

The current study highlighted the significance of β-lactam resistance through molecular and in silico characterization of β-lactamase enzyme in acute diarrheal isolates. Although *E. fergusonii* was first identified in 1985 and is well-known for triggering human, and animal-related infections and 64% genomic similarities with *E. coli*, very few earlier studies were executed on human samples to gain deeper insight into their pathogenicity in the global scenario, making this current one as the first-ever study from the North-Eastern part of India (Farmer et al., 1985). Similarly, *E. marmotae* is also a newly reported species from the faecal samples of a marmot species *Marmota himalayana*, which shows large variation with *E. coli* and *Shigella* species (Liu et al., 2015) and has been reported first time from this region. Moreover, in the phylogenetic relatedness study of the 29 isolates, the highest sequence similarities were observed with multi-continental non-Asian locations in cluster I, and both Asian as well as non-Asian isolation countries in clusters II & III (Figure 1).

The extended-spectrum behavior of antibiotic substrates among the resistance carrying isolates was observed by monitoring through MIC, phenotypic and genotypic tests, which can increase the risk of disease transmission. Isolates *E. fergusonii* S-10, *K. pneumoniae* S-2 and *S. dysenteriae* S-39 from, and species showed higher resistance tendency towards broad-spectrum carbapenem and cephalosporin group of antibiotics, thus expedite the need for further substantiate research on their genome characteristics. However, no earlier shreds of evidence on ESBL *bla*TEM from *E. fergusonii* and *E. marmotae* were reported from North-East India, marking this current finding as the first-ever study. Like the earlier findings, the current study also proposed the dominance of *Klebsiella* species carrying *bla*SHV gene variants in their chromosome (Rubin et al., 2020). Our study also reported, *bla*OKP-A (in *K. pneumoniae*) (Melano et al., 2006), *blaCTX-M-15* (in *E. fergusonii*) (Rubin et al., 2020), *bla*NDM (in *E. fergusonii*) (Bora et al., 2013; Choudhury et al., 2018; Devi et al., 2018; Ingti et al., 2018) (Rubin et al., 2020) and *bla*OXA (in *E. fergusonii* and *E. marmotae*) (Rubin et al., 2020) genes for the first time from North East India.

The chromosomally harbouring β-lactam resistance pattern of the enteric bacteria is often correlated with the mutational propensity of the β-lactamase enzymes that leads to alteration of β-lactam substrate activity. To the best of our knowledge, the non-catalytic substitutions at Arg 32, Leu 147 with the replacement of Ser, and Phe at the α-helix position in the non-cytoplasmic domain are some of the novel additions to the point mutation of BlaSHV enzyme enhancing the imipenem, ceftazidime, and cefpodoxime resistance in S-35 and S-46 isolates. Similarly, WT BlaSHV protein in S-9 and S-13 also carries Arg 32 and Leu 147, not as mutations but naturally found similarly as in their respective 100% homologs BlaSHV-28 and BlaSHV-63 respectively (Figure S13). Also, the study suggests that, in the S-46 strain, another firstly reported point mutation His92Tyr plays a foremost role in lowering the antibiotics resistance tendency of second and third-generation cephalosporin cefuroxime and ceftriaxone, compared to the S-35 strain. The amphipathic residue Tyr,
being on the surface of the protein, helps in protecting the membrane protein through regulating the substrate concentration in the cellular environment, hereby could be a potential factor for reducing the tendency of resistance in S-46 strain. Although, His and Tyr both correspond to an aromatic amino acid of the hydropathy class, the presence of phenol ring in Tyr make it accessible for stronger H-bond formation and thus in case of mutation at Tyr 92 position resulted in more favoured enzyme-substrate binding activity for lessening the antibiotic resistance. These two residues enhance the broad-spectrum activity of all the five afore-mentioned antibiotics in S-9 and S-13. Among the four blaSHV carrying isolates S-9, S-13, S-35 and S-46, S-9 exhibits the highest resistance profile (Table 1) with the conjugative effect of blaCTX-M in its chromosome. Conversely, among nine chromosomally encoded ESBL blaCTX-M genes, blaCTX-M of E. fergusonii S-10 strain were reported with a silent mutation of Val288Val (TCC→TCG6G in nucleotide position), which might partially restructure their evolutionary pattern as mentioned previously concerning ESBL enzymes.

In the local quality assessment of the model protein structure (x-axis), the comparative homology with the template target structure (y-axis), residues with <0.6, was defined as low quality score (Figure S18 & S19). The “degree of nativeness” of the modelled structure can be evaluated by QMEAN Z-score in the global estimation according to ExPasy analysis guidelines. For a reasonable comparison between X-ray crystallographic template and modelled protein structures, the value of the QMEAN Z-score should be around zero and the respective scores of −4.0 or less than it indicates poor quality of modelled proteins (Santhosh Kumar & Yusuf, 2020) and for S-9, S-13, S-35 and S-46 modelled BlaSHV protein structures, this score of greater than zero exhibited the good quality of structures. Besides, increasing resistance of imipenem, ceftazidime, cefuroxime, ceftriaxone and cefpodoxime in S-35 is associated with weak H-bond interaction of Arg 218, Ala 223, Asn 128, Ser 126, Gln 95, Asp 100, Tyr 101, Ser 102 and Ala 274 in BlaSHV binding pocket (Figure 7). Through our investigation, it can be assumed that the substitutions of polar and charged semi-essential amino acid Arg by polar and uncharged Ser could potentially decrease the overall substrate-enzyme interaction propensity, which leads to antibiotics resistance. In S-46, increasing resistance of imipenem, ceftazidime and cefpodoxime is influenced by the weak H-bond interaction of Asp 127, Ile 227, Arg 218, Asn 249, Thr 163, Asn 128, Ser 66, Ser 126 in BlaSHV binding pocket (Figure 8). In our observation, we have found that hydrophobic residue Phe147 mutation plays a foremost role by residing in the non-catalytic binding site of S-46 BlaSHV protein and correlating with the increasing substrate susceptibility. Although Ser 126, Asn 128 and Arg 218 residues were found common among the H-bonding
residues of mutant S-35 and S-46 BlaSHV protein-substrate complexes, they act differently by interacting with different substrates, perhaps due to additional mutation His92Tyr in S-46. In ceftazidime-SHV binding, Arg218, Ala223, Gly224, Trp225, Phe226, Ile227 and Asn249 were found as common binding sites residues in S-13 and S-46 strains. But, both the strains shared different resistance profiles, due to Tyr92 mutation in S-46. Cefpodoxime with its interaction to SHV resulted in the same binding site residues Ser66, Ser126, Thr163, Gly232, Gly234 in S-13 and S-46, Gly234 in S-13 and S-35 and Ala233, Gly234 in S-35 and S-46 with low to moderate binding affinity and make all the four strains resistant. In the case of cefoxitin-SHV interaction, the reason behind the sensitiveness is due to Val220 binding site in S-9 and S-35 and Ser126, Ala233 in S-13 and S-46 strain. For Ceftriaxone- SHV interaction, although Ile227, Val208, Phe226 in S-13 and S-46 and Leu221 in S-35 and S-46 were common binding residues, the His92Tyr mutation in S-46 leads to its sensitiveness towards the antibiotics. Another second-generation cephalosporin Cefuroxime on its binding with SHV protein found to be shared similar binding sites Gln205, Val208 and Phe226 in S-9 and S-13 WT strains making it highly resistant. Although, mutant strain S-35 did not share any of these binding sites, still showed higher resistance to Cefuroxime. In the case of Doripenem-SHV interaction, Ser66, Asn128, Tyr101, Ala233, Gly234 active site residues trigger sensitivity in S-13 and S-35 strains. On the other hand, S-9 and S-46 were also found to be sensitive to the influence of unique binding site residues as shown in Table 2. For Ertapenem-SHV interaction, S-9 and S-46 share the same binding site residues such as Ala75, Val76, Ala78, Val138 and Gly139 with moderate binding affinity to make them sensitive towards the antibiotic. In highly resistant imipenem-SHV interaction, Met65, Ser66, Ser126, Thr231 was common in S-9 and S-13, Tyr101, Asp127, Gly232, Ala233 in S-9 and S-46 and Lys69, Gly232, Ala233 in S-13 and S-46 with very low binding affinity, hence assisting in higher resistance profile. In meropenem- SHV interaction, we could able to see the high binding affinity but, due to the presence of BlaCTX-M in S-9, it exhibited higher meropenem resistance over all the three sensitive strains. The predominance of hydrophobic amino residues found in the active site and its neighbouring sites demonstrated the correlation with mounting resistance among BlaSHV carrying strains.

**Conclusions**

β-lactam resistance has emerged as a global burden with the significant diversity among the resistance causing Enterobacteriaceae bacteria. Owing to the novel mutational tendency of β-lactamase encoding genes, extensive spectrum activity of β-lactam antibiotics can be observed frequently. Throughout these findings, the first-ever report of *E. fergusonii* and *E. marmotae* strains from acute diarrheal patients of

**Figure 8.** SHV-11 enzyme of S-46 + β-lactam substrate complexes obtained from molecular docking study where (a-i) represents cefoxitin, cefpodoxime, ceftazidime ceftriaxone, cefuroxime, doripenem, ertapenem, imipenem and meropenem binding site with corresponding amino acid residues.
North East India can be highlighted for which substantial study could be implemented further. Chromosomal encoding \( \beta \)-lactamase genes blaOKP-A is also reported first time from this region by specifying their role in the dissemination of resistance during acute diarrheal diseases. The current study reports the three novel mutations Arg32Ser, His92Tyr, and Leu147Phe in BlaSHV proteins which have an essential role in antibiotics resistance diversity. Overall, in these findings, Phe 147 being a substitute residue in mutant and a naturally exist residue in WT strain, plays a chief role in increasing resistance pattern. Irrespective of carrying some similar binding site residues with other three resistant strain S-5, S-13 and S-35, His92Tyr mutation possess a great role in minimizing resistance in S-46 strain, which is a matter of concern for more substantial research. The correlation between enzyme-substrate interaction and antibiotics substrate resistance is also observed through molecular docking study. Henceforth, to achieve better therapeutics, the wide-ranging geographical distribution of \( \beta \)-lactamase genes must be studied in well means by identifying various unique mutations.

Acknowledgements

We thank the Director, CSIR-North East Institute of Science and Technology (CSIR-NEIST), Jorhat, India, for his consistent encouragement and support.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

S.S. is supported by DST-INSP IRE fellowships (IF-170007) and B.D is supported by DST-SERB. This work was funded by the Council of Scientific and Industrial Research (CSIR), New Delhi (OLP-2035) and Department of Science and Technology-SERB (GP P-0329) to AKS.

ORCID

Anil Kumar Singh http://orcid.org/0000-0003-0375-9242

References

Bennett, P. M. (2008). Plasmid encoded antibiotic resistance: Acquisition and transfer of antibiotic resistance genes in bacteria. British Journal of Pharmacology, 153(S1), S347–S357. https://doi.org/10.1038/sj.bjp.0707607

Bora, A., Ahmed, G. U., Hazarika, N. K., Prasad, K. N., Shukla, S. K., Randhawa, V., & Sarma, J. B. (2013). Incidence of blaNDM-1 gene in Escherichia coli isolates at a tertiary care referral hospital in Northeast India. Indian Journal of Medical Microbiology, 31(3), 250–256. https://doi.org/10.4103/0255-0857.115628

Bradford, P. A. (2001). Extended-spectrum \( \beta \)-lactamas in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clinical Microbiology Reviews, 14(4), 933–951. https://doi.org/10.1128/cmrr.14.4.933-951.2001

Buchan, D. W. A., & Jones, D. T. (2019). The PSIPRED protein analysis workbench: 20 years on. Nucleic Acids Research, 47(W1), W402–W407.

Bush, K., & Fisher, J. F. (2011). Epidemiological expansion, structural studies, and clinical challenges of new \( \beta \)-lactamases from gram-negative bacteria. Annual Review of Microbiology, 65, 455–478.

Choudhury, N. A., Paul, D., Chakravarty, A., Bhattacharjee, A., & Chanda, D. D. (2018). IncX3 plasmid mediated occurrence of blaNDM-4 within Escherichia coli ST448 from India. Journal of Infection and Public Health, 11(1), 111–114.

Ciccozzi, M., Cella, E., Lai, A., De Florio, L., Antonelli, F., Fogolari, M., Di Matteo, F. M., Pizzicannella, M., Colombo, B., Dicuonzo, G., & Angeletti, S. (2019). Phylogenetic analysis of multi-drug resistant Klebsiella pneumoniae strains from duodenumoscope biofilm: Microbiological surveillance and reprocessing improvements for infection prevention. Frontiers in Public Health, 7, 219. https://doi.org/10.3389/fpubh.2019.00219

Dallenne, C., Da Costa, A., Decré, D., Favier, C., & Arlet, G. (2010). Development of a set of multiplex PCR assays for the detection of genes encoding important \( \beta \)-lactamases in Enterobacteriaceae. Journal of Antimicrobial Chemotherapy, 65(3), 490–495. https://doi.org/10.1093/jac/dkp498

Datta, S., Wattal, C., Goel, N., Oberoi, J. K., Raveendran, R., & Prasad, K. J. (2012). A ten year analysis of multi-drug resistant blood stream infections caused by Escherichia coli & Klebsiella pneumoniae in a tertiary care hospital. The Indian Journal of Medical Research, 135(6), 907.

Devi, U., Bora, R., Das, J. K., & Mahanta, J. (2018). Extended-spectrum \( \beta \)-lactamase & carbapenemase-producing gram-negative bacilli in neonates from a tertiary care Centre in Dibrugarh, Assam, India. The Indian Journal of Medical Research, 147(1), 110–114. https://doi.org/10.4103/ijmr.IJMR_1288_16

Dharla, L., Tripathi, A., & Pal, A. (2013). Molecular characterization and in silico analysis of naturally occurring TEM beta-lactama variants among pathogenic Enterobacteriaceae infecting Indian patients. BioMed Research International, 2013, 783540. https://doi.org/10.1155/2013/783540

Evans, B., & Amyes, S. (2014). Oxa \( \beta \)-lactamase. Clinical Microbiology Reviews, 27(2), 241–263. https://doi.org/10.1128/CMR.00117-13

Farmer, J. J., Fanning, G. R., Davis, B. R., O’Hara, C. M., Riddle, C., Hickman-Brenner, F. W., Asbury, M. A., Lowery, V. A., & Brenner, D. J. (1985). Escherichia fergusonii and Enterobacter taylorae, two new species of Enterobacteriaceae isolated from clinical specimens. Journal of Clinical Microbiology, 21(1), 77–81.

Garau, J. (1994). Beta-lactamas: Current situation and clinical importance. Intensive Care Medicine, 20(3), 55–59. https://doi.org/10.1007/BF01745244

Gasteiger, E., Hoogland, C., Gattiker, A., Wilkins, M. R., Appel, R. D., & Bairoch, A. (2003). Protein identification and analysis tools on the ExPaSy server (pp. 571–607). The Proteome Protocols Handbook. Geourjon, C., & Deleage, G. (1995). SOPMA: Significant improvements in protein secondary structure prediction by consensus prediction from multiple alignments. Computer Applications in the Biosciences: CABIOS, 11(6), 681–684. https://doi.org/10.1093/bioinformatics/11.6.681

Ingli, B., Saikia, P., Paul, D., Maurya, A. P., Dhar, D., & Chakravarty, A. (2018). Occurrence of blaCMY-42 through IncI1 plasmid within multi-drug-resistant Escherichia coli from a tertiary referral hospital of India. Journal of Global Antimicrobial Resistance, 14, 78–82.

Jeon, A. B., Obregón-Henao, A., Ackart, D. F., Podell, B. K., Belardelli, J. M., Jackson, M., Nguyen, T. V., Blackledge, M. S., Melander, R. J., Melander, C., Johnson, B. K., Abramovitch, R. B., & Basaraba, R. J. (2017). 2-aminomimidazoles potentiate \( \beta \)-lactam antimicrobial activity against Mycobacterium tuberculosis by reducing \( \beta \)-lactamate secretion and increasing cell envelope permeability. PloS One, 12(7), e0180925. https://doi.org/10.1371/journal.pone.0180925

Jones, D. T. (1999). Protein secondary structure prediction based on position-specific scoring matrices. Journal of Molecular Biology, 292(2), 195–202. https://doi.org/10.1006/jmbi.1999.3091

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. Molecular Biology and Evolution, 35(6), 1547–1549.

Ling, B.-D., Liu, G., Xie, Y.-E., Zhou, Q.-X., Zhao, T.-K., Li, C.-Q., Yu, X., & Lei, J. (2006). Characterisation of a novel extended-spectrum...
β-lactamase, SHV-70, from a clinical isolate of Enterobacter cloacae in China. Liu, S., Jin, D., Lan, R., Wang, Y., Meng, Q., Dai, H., Lu, S., Hu, S., & Xu, J. (2015). Escherichia marmota sp. nov., isolated from faeces of Marmota himalayana. *International Journal of Systematic and Evolutionary Microbiology*, 65(7), 2130–2134.

Livermore, D. M., & Hawkey, P. M. (2005). CTX-M: Changing the face of ESBLs in the UK. *Journal of Antimicrobial Chemotherapy*, 56(3), 451–454. https://doi.org/10.1093/jac/dki239

Medjehed, H., & Singh, A. K. (2010). Genetic manipulation of Mycobacterium abscessus. *Current Protocols in Microbiology*.

Mishra, S., Sen, M. R., Upadhyay, S., & Bhattacharjee, A. (2013). Genetic linkage of blaNDM among nosocomial isolates of Acinetobacter baumanii from a tertiary referral hospital in northern India. *International Journal of Antimicrobial Agents*, 41(5), 452–456.

Mulani, M. S., Kamble, E. E., Kumkar, S. N., Tawre, M. S., & Pardesi, K. R. (2019). Emerging strategies to combat ESKAPE pathogens in the era of antimicrobial resistance: A review. *Frontiers in Microbiology*, 10, 539.

Niumsup, P., Simm, A. M., Nurmahomed, K., Walsh, T. R., Bennett, P. M., & Avison, M. B. (2003). Genetic linkage of the penicillinase gene, amp, and blrAB, encoding the regulator of β-lactamase expression in Aeromonas spp. *Journal of Antimicrobial Chemotherapy*, 51(6), 1351–1358. https://doi.org/10.1093/jac/dkg247

Palzkill, T. (2018). Structural and mechanistic basis for extended-spectrum drug-resistance mutations in altering the specificity of TEM, CTX-M, and KPC β-lactamases. *Frontiers in Molecular Biosciences*, 5, 16. https://doi.org/10.3389/fmolb.2018.00016

Pasteran, F., Tijet, N., Melano, R. G., & Corso, A. (2015). Simplified protocol for Carba NP test for enhanced detection of carbapenemase producers directly from bacterial cultures. *Journal of Clinical Microbiology*, 53(12), 3908–3911.

Philippon, A., Slama, P., Déný, P., & Labia, R. (2016). A structure-based classification of class A β-lactamases, a broadly diverse family of enzymes. *Clinical Microbiology Reviews*, 29(1), 29–57. https://doi.org/10.1128/CMR.00019-15

Rubin, J., Mussio, K., Xu, Y., Suh, J., & Riley, L. W. (2020). Prevalence of antimicrobial resistance genes and integrons in commensal Gram-negative bacteria in a college community. *Microbial Drug Resistance*, 26(10), 1227–1235. https://doi.org/10.1089/mdr.2019.0279

Santhosh Kumar, R., & Yusuf, A. (2020). In silico structural modeling and analysis of physicochemical properties of curcumin synthase (CURS1, CURS2, and CURS3) proteins of Curcuma longa. *Journal of Genetic Engineering and Biotechnology*, 18(1), 1–9. https://doi.org/10.1186/s43141-020-00041-x

Savard, P., & Perl, T. M. (2012). A call for action: Managing the emergence of multidrug-resistant Enterobacteriaceae in the acute care settings. *Current Opinion in Infectious Diseases*, 25(4), 371–377. https://doi.org/10.1097/QCO.0b013e3283558c17

Stavri, M., Schneider, R., O’Donnell, G., Lechner, D., Bucar, F., & Gibbons, S. (2004). The antimycobacterial components of hops (Humulus lupulus) and their dereplication. *Phytotherapy Research: PTR*, 18(9), 774–776. https://doi.org/10.1002/ptr.1527

Tamura, K., & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution*, 10(3), 512–526.

Walter-Rasmussen, J., & Haiby, N. (2002). Plasmid-borne AmpC β-lactamases. *Canadian Journal of Microbiology*, 48(6), 479–493.

Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., Heer, F. T., de Beer, T. A. P., Rempfer, C., Bordoli, L., Lepore, R., & Schwede, T. (2018). SWISS-MODEL: Homology modelling of protein structures and complexes. *Nucleic Acids Research*, 46(W1), W296–W303.

World Health Organization. (2019). WHO publishes list of bacteria for which new antibiotics are urgently needed. 2017.