Molecular Analysis of ß-Lactamase Genes to Understand their Differential Expression in Strains of *Yersinia enterocolitica* Biotype 1A

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Beta-lactams are used as major therapeutic agents against a number of infectious agents. Due to widespread use of ß-lactams, ß-lactamases have evolved at a rapid pace leading to treatment failures. *Yersinia enterocolitica* causes many gastrointestinal problems. It is an extremely heterogeneous species comprising more than fifty serotypes and six biotypes which differ in their ecological niches, geographical distribution and pathogenic potential. Though biotype 1A strains have been associated with outbreaks of Yersiniosis, there has been a controversy regarding their pathogenicity. The strains of *Y. enterocolitica* isolated from India belonged to biotype 1A and possessed genes for two ß-lactamases namely, blaA and blaB. An earlier study by us reported differential expression of blaA by strains of *Y. enterocolitica* biotype 1A. The present study has been carried out to understand the molecular bases which regulate the expression of blaA in *Y. enterocolitica* biotype 1A. We concluded that six types of blaA variants were present in strains of biotype 1A. Neither amino acid substitutions in blaA nor mutations in promoter regions of blaA contributed to differential expression of blaA in *Y. enterocolitica* biotype 1A. Rather, the secondary structures attained by mRNA of blaA might underlie the differential expression of blaA in *Y. enterocolitica*.

Beta-lactamases and their mutated forms such as extended spectrum ß-lactamases (ESBLs), AmpC ß-lactamases, carbapenemases and metallo ß-lactamases represent a major cause of multidrug resistance among enteric bacteria. Following selective pressure due to widespread use of ß-lactam antibiotics in clinical medicine, genes for ß-lactamases have evolved at a very rapid pace with over 1300 unique ß-lactamases reported in clinical isolates¹. *Yersinia enterocolitica*, a member of the family *Enterobacteriaceae* is an important zoonotic pathogen. It is an extremely heterogeneous species comprising fifty serotypes and six biotypes viz. 1A, 1B, 2, 3, 4 and 5 which differ not only in their geographical distribution and ecological niches but also in pathogenic potential². On the basis of pathogenicity, *Y. enterocolitica* can be divided into highly pathogenic biotype 1B strains, moderately pathogenic biotype 2–5 strains and supposedly non-pathogenic biotype 1A strains. Batzilla et al.³ proposed that biotype 1A strains represent a potential group of emerging pathogens which share known and putative virulence-associated features with the pathogenic bioserotypes. Studies have shown that strains classified as biotype 1A, in fact, represent more than one subspecies⁴. Despite the lack of classical determinants of virulence in biotype 1A strains⁴ these have been frequently isolated from clinical samples across the globe⁵⁻⁷.

In India, the first food-borne outbreak due to *Y. enterocolitica* was reported from Tamil Nadu in 1997⁸. Since then, this pathogen has been isolated from a variety of sources – wastewater, pork, pigs, diarrheic human patients, ice cream, dairy products and even traditional fast food⁹⁻¹³. The strains of *Y. enterocolitica* isolated from India⁶,¹¹ principally belonged to biotype 1A. The strains of *Y. enterocolitica* are known to possess two ß-lactamases, namely ß-lactamase A (blaA) and ß-lactamase B (blaB)¹⁴,¹⁵. BlaA is a broad-spectrum constitutively expressed Ambler class A enzyme where as blaB is an inducible Ambler Class C enzyme¹⁶.

The most common mode of microbial resistance to ß-lactams is their enzymatic hydrolysis by ß-lactamases. Over the time, ß-lactamases have expanded their substrate spectrum with simple mutations in the gene or in the genetic environment of ß-lactamases. Modifications in the regulatory regions, mutations in the promoter sequences and integration of insertion sequences containing efficient promoters have frequently been associated
with high-level expression of chromosomal β-lactamases. Studies on bla genes of Y. enterocolitica have reported differences in β-lactamase expression and β-lactam susceptibility among different biotypes, focusing primarily on pathogenic biotypes. The previous study on molecular characterization of bla genes and differences in β-lactamase expression in biotype 1A strains was reported from our laboratory. Previous studies from our laboratory also reported that the expression of blaB in biotype 1A strains was quite similar among the strains, while the expression of blaA was quite variable among different strains. To the best of our knowledge, till date no study has been carried out to understand the differential regulation of expression of blaA in Y. enterocolitica biotype 1A strains. The present study is an extension of our previous observations and was undertaken to understand the molecular basis of differential expression of β-lactamase genes of biotype 1A strains of Y. enterocolitica. This study also provides insights into the blaA variants in biotype 1A strains of Y. enterocolitica. Thus, the promoter regions and the structural regions of blaA genes in biotype 1A strains of Yersinia enterocolitica isolated from India and from other parts of the world were PCR-amplified, sequenced and compared. Three dimensional structures of blaA variants were modeled in-silico to co-relate the role of specific substitutions in differential expression of β-lactamases.

**Results**

**Minimal Inhibitory Concentrations of β-lactam antibiotics.** Y. enterocolitica strains showed variable susceptibilities to β-lactam antibiotics of different generations (Table 1). All biotype 1A strains of Indian and non-Indian origin except Y. enterocolitica strain E3 were resistant to amoxicillin and amoxicillin-clavulanate, antibiotic-inhibitor combination. The MIC of all biotype 1A strains to amoxicillin exceeded the upper range of E-test i.e >256 mg/L. For six Indian strains, the MIC of amoxicillin-clavulanate combination also exceeded the upper range of E-test. The susceptibility of Y. enterocolitica to the third generation cephalosporins, cefpodoxime and cefotaxime was also tested by E-test. Antibiotic susceptibility of Y. enterocolitica strains to the third generation cephalosporin cefpodoxime varied and ranged from 0.125–8 mg/L. While most of the biotype 1A strains were either sensitive or intermediate to cefpodoxime, four strains were resistant to this antibiotic. Further, all Y. strains were sensitive to third generation cephalosporin, cefotaxime. For biotype 1A strains the MIC was lowest for cefotaxime and ranged between 0.032–0.64 mg/L. Strain E3 showed lowest MIC to all antibiotics including amoxicillin and amoxicillin-clavulanate.

**Specific activities of β-lactamases.** The mean specific activities of the blaA variants produced by Y. enterocolitica biotype 1A strains expressed as amount of nitrocefin hydrolyzed (μmol/min/mg of protein) are summarized in Table 2. The specific activity of the variant blaA2 was quite high than the mean specific activities of other variants, while that of blaA4 was the least. The mean specific activities of the variants blaA1 and blaA3 were similar to each other.

**Promoter sequences and CCDs of blaA gene.** The amplification of blaA gene using primers blaA11 and blaA12 resulted in the expected amplicon of 1.49 kb in 68 out of 81 strains of Y. enterocolitica. The band was excised from the agarose gel and sequenced after purification using QIAGEN purification kit. The BLAST analysis of the sequences confirmed these to be part of the blaA gene. Multiple sequence alignment of the promoter regions revealed that promoter sequences of all biotype 1A strains were identical (Fig. 1).

| Table 1 | MIC of selected antibiotics for Y. enterocolitica biotype 1A strains |
|---|---|---|---|---|---|---|
| Y.e strain 1 | Clinical | India | O:6,30–6,31 | 256(R) | >256(R) | 0.64(S) | 1(S) |
| Y.e strain 2 | Clinical | India | O:6,30–6,31 | 256(R) | >256(R) | 0.125(S) | 2(S) |
| Y.e strain 3 | Clinical | India | O:6,30–6,31 | 256(R) | 192(R) | 0.036(S) | 0.75(S) |
| Y.e strain 8 | Clinical | India | O:6,30–6,31 | 256(R) | 128(R) | 0.125(S) | 2(S) |
| Y.e strain 9 | Wastewater | India | O:6,30–6,31 | 256(R) | 128(R) | 0.19(S) | 3(S) |
| Y.e strain 10 | Wastewater | India | O:6,30–6,31 | 256(R) | 192(R) | 0.38(S) | 3(S) |
| Y.e strain 11 | Wastewater | India | O:6,30–6,31 | 256(R) | 64(R) | 0.75(S) | 8(R) |
| Y.e strain 12 | Clinical | India | O:6,30–6,31 | 256(R) | 128(R) | 0.19(S) | 8(R) |
| Y.e strain 19 | Clinical | India | O:6,30 | 256(R) | >256(R) | 0.125(S) | 3(S) |
| Y.e strain 20 | Clinical | India | O:6,30 | >256(R) | 0.064(S) | 1(S) |
| Y.e strain 28 | Wastewater | India | O:15 | >256(R) | 0.64(S) | 1(S) |
| Y.e strain 29 | Wastewater | India | O:41,42 | >256(R) | 192(R) | 0.125(S) | 2(S) |
| Y.e strain 30 | Clinical | India | O:41,43 | >256(R) | 128(R) | 0.125(S) | 0.3(S) |
| Y.e strain 35 | Pig throat | India | ND | >256(R) | 192(R) | 0.125(S) | 2(S) |
| Y.e strain 42 | Clinical | India | O:6,30 | >256(R) | 0.094(S) | 2(S) |
| Y.e strain 47 | Clinical | India | O:6,30 | >256(R) | 128(R) | 0.25(S) | 4(S) |
| Y.e strain 52 | Wastewater | India | O:6,30–6,31 | >256(R) | >256(R) | 0.25(S) | 4(S) |
| Y.e strain 109 | Clinical | India | NAG | >256(R) | 192(R) | 0.25(S) | 3(S) |
| Y.e strain 111 | Clinical | India | NAG | >256(R) | 192(R) | 0.25(S) | 3(S) |
| Y.e strain E2 | Clinical | Germany | O:6,30 | >256(R) | 128(R) | 0.75(S) | 8(R) |
| Y.e strain E3 | Clinical | Germany | O:6,30 | 64(S) | 0.75(S) | 0.032(S) | 0.125(S) |
| Y.e strain E4 | Clinical | NK | O:6,30 | >256(R) | 128(R) | 0.75(S) | 8(R) |
| Y.e strain E7 | Clinical | France | O:6,30 | >256(R) | 192(R) | 0.38(S) | 2(S) |
| Y.e strain E8 | Clinical | France | O:6,30 | >256(R) | 64(R) | 0.75(S) | 4(S) |

AMX, amoxicillin; AMC, amoxicillin/clav; CTX, cefotaxime; CPD, cefpodoxime; Alphabets in parenthesis indicate antibiotic susceptibility; R-resistant, I-intermediate, S-sensitive. ND= not determined; NAG= non agglutinable; NK= not known.
35 strains viz. *Y. enterocolitica* strains 12, 52, 8, 42, 47, 48, 109, 111, E8, 10, 11; G58A in *Y. enterocolitica* strains E2, E4, E3, 29, 30, E7; R69Q in *Y. enterocolitica* strains E6, E2, E4, E3, 29, 30, E7; I98T and N101D in *Y. enterocolitica* strain 19. I113V and T225S in *Y. enterocolitica* strain E4; L144V in *Y. enterocolitica* strains E2 and E4. The mutation sites in individual strains are shown in Table 3 and summarized in Fig. 2.

Phylogenetic analysis of blaA of *Y. enterocolitica* strains. The phylogenetic analysis of blaA of 35 strains of *Y. enterocolitica* biotype 1A was carried using the Maximum Likelihood method of MEGA5.5. The tree with the highest log likelihood (−939.3021) is shown (Fig. 3). The tree is drawn to scale, with branch lengths

| Table 2 | Mean specific activities of β-lactamase (blaA) variants of *Y. enterocolitica* biotype 1A strains |
|---|---|
| blaA variant | No. of strains in which the variant was present (n) | Mean specific activity (μmol/min/mg of protein) of β-lactamase |
| blaA1 | 12 | 0.150 ± 0.02 |
| blaA2 | 1 | 0.853 |
| blaA3 | 16 | 0.153 ± 0.04 |
| blaA4 | 4 | 0.030 ± 0.02 |
| blaA5 | 1 | 0.10 |
| blaA6 | 1 | 0.20 |

All values are represented as mean ± standard error of mean (SEM).

Y. *enterocolitica* strain 1
Y. *enterocolitica* strain 18
Y. *enterocolitica* strain E1
Y. *enterocolitica* strain 103
Y. *enterocolitica* strain 46
Y. *enterocolitica* strain E9
Y. *enterocolitica* strain 7
Y. *enterocolitica* strain 19
Y. *enterocolitica* strain 20
Y. *enterocolitica* strain 2
Y. *enterocolitica* strain 107
Y. *enterocolitica* strain E10
Y. *enterocolitica* strain 13
Y. *enterocolitica* strain E16
Y. *enterocolitica* strain E15
Y. *enterocolitica* strain E11
Y. *enterocolitica* strain 43
Y. *enterocolitica* strain 3
Y. *enterocolitica* strain E12
Y. *enterocolitica* strain 106
Y. *enterocolitica* strain 17
Y. *enterocolitica* strain 22
Y. *enterocolitica* strain 4
Y. *enterocolitica* strain 16
Y. *enterocolitica* strain 45
Y. *enterocolitica* strain 47
Y. *enterocolitica* strain E4
Y. *enterocolitica* strain 44
Y. *enterocolitica* strain 21
Y. *enterocolitica* strain 109
Y. *enterocolitica* strain 110
Y. *enterocolitica* strain 111
Y. *enterocolitica* strain E7
Y. *enterocolitica* strain E8
Y. *enterocolitica* strain 104
Y. *enterocolitica* strain 42
Y. *enterocolitica* strain 41
Y. *enterocolitica* strain E4
Y. *enterocolitica* strain E5
Y. *enterocolitica* strain E2
Y. *enterocolitica* strain E3
Y. *enterocolitica* strain 48

Figure 1 | Multiple sequence alignment of the promoter region of blaA of representative strains of *Y. enterocolitica*. The transcription start site ATG, −10 region and −30 regions are shown in bold face; −10 and −30 regions were highly conserved in all the strains.
represented by the number of substitutions per site. The MEGA analysis clustered isolates into three major groups.

**Modeling analysis.** Based on the identity percentage and E-values (expectation values), β-lactamase of *Burkholderia multivorans* (PDB code 3W4Q-A; Uniprot ID A9ANW2) was selected as the template protein due its high sequence similarity, identity and low E-value. Of the 20 models built using MODELLER, the 3D model with the lowest modeler objective function was selected as the final model.

**Protein structure validation by PROCHECK and VERIFY3D.** The results of PROCHECK as represented in Ramachandran contour plot showed that ca. 90% of the residues lay in the most favoured regions and only 1% of residues in disallowed regions. Verify3D profiles which were used to identify unreliable regions that had been modeled improperly showed that 90% region of the protein model of each blaA variant scored > 0.2 which was highly significant.

**Molecular docking.** Evaluation of binding affinity of β-lactam antibiotics with class A β-lactamas was performed using AutoDock Vina. The binding poses for each ligand molecule (amoxicillin, clavulanic acid) into the structures were determined and different poses were generated based on the total score (Dock score). The negative low free energy of binding of docked complexes and low inhibition constants indicated high affinity of β-lactamase for these antibiotics (Table 4 & 5, Fig. 4).

**mRNA secondary structure analysis.** The secondary structures of mRNA of the six blaA variants were analyzed. These formed branched secondary structures. The structure of blaA2 showed very high level of branching, with a number of bulge loops, interior loops and a smaller region of double stranded RNA helix. On the other hand the mRNA secondary structure of blaA4 was mostly composed of a double-stranded RNA helix of stacked base pairs with little branching (Fig. 5).

| Sr. No. | Strain designation | Source | Serotype | Amino acid change |
|--------|-------------------|--------|----------|------------------|
| 1. | *Y. enterocolitica* strains 8, 9, 10, 11, 12, 52 | Wastewater (India) | O:6,30–6,31 | P32S |
| 2. | *Y. enterocolitica* strains 42, 47, 48, E8* | Human stools (India)* Human (Europe) | O:6,30 | P32S |
| 3. | *Y. enterocolitica* strains 109, 111 | Human stools (India) | NAG | P32S |
| 4. | *Y. enterocolitica* strains E2, E3, E4, E7 | Human (Europe) | O:6,30 | G58A |
| 5. | *Y. enterocolitica* strains 29, 30 | Wastewater (India) | O:41,42 | G58A |
| 6. | *Y. enterocolitica* strains 8, 9, 10, 11, 12, 52 | Wastewater (India) | O:6,30–6,31 | G60C |
| 7. | *Y. enterocolitica* strains 42, 47, 48, E8* | Human stools (India)* Human (Europe) | O:6,30 | G60C |
| 8. | *Y. enterocolitica* strains 109, 111 | Human stools (India) | NAG | G60C |
| 9. | *Y. enterocolitica* strains 29, 30 | Wastewater (India) | O:41,42 | R69Q |
| 10. | *Y. enterocolitica* strains E2, E3, E4, E6, E7 | Human (Europe) | O:6,30 | R69Q |
| 11. | *Y. enterocolitica* strain 19 | Wastewater (India) | O:6,30–6,31 | L98T |
| 12. | *Y. enterocolitica* strain 19 | Wastewater (India) | O:6,30–6,31 | N101D |
| 13. | *Y. enterocolitica* strain E4 | Human (Europe) | O:6,30 | I113V |
| 14. | *Y. enterocolitica* strains E2, E4 | Human (Europe) | O:6,30 | L144V |
| 15. | *Y. enterocolitica* strain E4 | Human (Europe) | O:6,30 | T225S |

**Discussion**

The MIC of different β-lactam antibiotics for *Y. enterocolitica* biotype 1A strains using E-test revealed that all strains were resistant to amoxicillin and amoxicillin-clavulanate. *Y. enterocolitica* strains have been reported to be ‘naturally resistant’ to ampicillin30,31. Although the MIC of amoxicillin-clavulanate was perceptibly less than that of amoxicillin, it did not change the resistance phenotype, implying the existence of inhibitor resistant blaAs in *Y. enterocolitica* biotype 1A. Earlier studies have shown that *Y. enterocolitica* strains of serotypes O: 3, O: 5 and O: 931 were all resistant to ampicillin and a few were resistant to amoxicillin-clavulanate. Recent studies32,33 also showed that strains of *Y. enterocolitica* bioserotype 4/ O: 3 were sensitive to amoxicillin-clavulanate where as that of bioserotypes 2/O: 5, 27 and 2/O: 9 were resistant. From the available information it is apparent that both the biotype and serotype play role in affecting the susceptibility of *Y. enterocolitica* to amoxicillin-clavulanate. Since all strains in the present study belonged to biotype 1A, it is not possible to comment on the influence of biotype on antibiotic susceptibility; but based on our findings it may be concluded that serotype had no effect on the susceptibility of *Y. enterocolitica* biotype 1A strains to amoxicillin-clavulanate. The biotype 1A strains were either sensitive or showed intermediate susceptibility to the third generation cephalosporin, cefpodoxime and only a few strains were resistant. This is the same as observed in an earlier study34. *Y. enterocolitica* strain E3 was sensitive to all the β-lactam antibiotics tested and showed lowest MIC to all the β-lactam antibiotics tested including amoxicillin and amoxicillin-clavulanate combination.

The mean specific activities of variants blaA1 and blaA3 were almost similar and the mutations/substitutions which lead to these variants had no effect on the enzyme activity. Similarly mutations that generated variants blaA5 and blaA6 did not affect the enzyme activity.
activity significantly. However, the mutations leading to variants blaA2 and blaA4 significantly increased or decreased the specific activities of the enzyme respectively.

Bacteria have been reported to hyper produce β-lactamases by different mechanisms viz. mutation in the promoters, integration of insertion sequences containing efficient promoters or point mutations in the structural gene. In the family Enterobacteriaceae, mutations and insertions, mostly in the promoter regions of the β-lactamase genes, have been associated with hyper production of β-lactamases. In the present study, we attempted to understand the molecular basis of differential expression of chromosomal blaA of biotype 1A strains of Y. enterocolitica. For this, the nucleotide sequences of promoter regions of blaA of biotype 1A strains were PCR-amplified and analyzed. Genetic events that affect the transcription efficacy of bacterial promoters usually occur in the 235 or 210 regions or sometimes in the space between these regions. It is well known that promoter strength is linked to the sequence of the 235 and 210 boxes, creating the 70 bp contact region for RNA polymerase. The 210 sequence TATAAT and the 235 sequence GTGGTG of blaA of Y. enterocolitica were similar to the canonical E.coli sequence indicating these were strong promoters. Alignment and comparison of blaA of biotype 1A strains by Clustal revealed that the ribosomal binding site (RBS), the 210 region and 235 regions were identical in all strains indicating that the promoter region had no role in differential expression of β-lactamases. Thus it was argued that the differential expression of class A beta-lactamase enzyme in Y. enterocolitica biotype 1A might be due to the changes in gene sequence of blaA.

To investigate the role of changes in gene sequence of blaA in differential expression of enzyme, blaA genes of biotype 1A strains were PCR-amplified and their CCDs translated into corresponding amino acid sequences. The four significant motifs conserved across the Ambler class A β-lactamase viz. SXXK, SDN, EXXLN and KTG, (where X represents any amino acid) were present in blaA of all Y. enterocolitica strains, their respective sequence and position being 75STFK78, 136SDN138, 171EPDLN175 and 239KTG241. One of the four conserved domains 171EPDLN175 was located in the omega loop, a structural domain constituting a part of active-site pocket. Clustal alignment and comparison of amino acid sequences of structural β-lactamase blaA of biotype 1A strains revealed that amino acid substitutions were present at some places. Since the initial 30 amino acids constitute the signal sequence and are cleaved before the

| Table 4 | Estimated inhibition constants, free energy of binding, H-bond interactions and hydrophobic interactions between amoxicillin and blaA variants of Y. enterocolitica biotype 1A |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| blaA variant    | Estimated Inhibition Constant, Ki (μM) | Free Energy of Binding (kcal/mol) | Interacting Residues |
| blaA1           | 57.12           | –5.79           | Hbonds: 75S, 135S, 137N, 175N, 242S |
|                 |                 |                 | Hydrophobic interactions: 78K, 109S, 110Y, 171E, 172P, 221T, 243G, 244D, 279R |
| blaA2           | 14.30           | –6.61           | Hbonds: 75S, 78K, 135S, 221T, 242S, 279R |
|                 |                 |                 | Hydrophobic interactions: 74C, 109S, 110Y, 137N, 171E, 172P, 175N, 240T, 241G |
| blaA3           | 4.31            | –7.32           | Hbonds: 75S, 109S, 135S, 137N, 171, 242, 279 |
|                 |                 |                 | Hydrophobic interactions: 78K, 110Y, 134Y, 175N, 221T, 240T |
| blaA4           | 21.79           | –6.36           | Hbonds: 135S, 137N, 175N, 240T, 242S |
|                 |                 |                 | Hydrophobic interactions: 75S, 78K, 109S, 110Y, 171E, 221T, 241G, 243G |
| blaA5           | 116.57          | –5.37           | Hbonds: 75S, 109S, 135S, 137N, 175N, 242S |
|                 |                 |                 | Hydrophobic interactions: 78K, 110Y, 171E, 221T, 243G |
| blaA6           | 15.17           | –6.57           | Hbonds: 75S, 135S, 137N, 171H, 242S, 279H |
|                 |                 |                 | Hydrophobic interactions: 78K, 110Y, 175N, 221T, 240T |
mature enzyme is released in the periplasmic space, these were excluded from the comparative studies. The most frequent point substitutions observed in 12 of the 35 *Y. enterocolitica* strains were a substitution from P32S and G60C. As proline and serine are both polar amino acids, substitution of one with the other will affect only the bulkiness of the side chain of the protein. Glycine is a simple nonpolar amino acid, its replacement with cysteine, a polar sulfur containing amino acid may affect the properties of the side chain of the protein. The substitution of glycine with alanine G58A in *Y. enterocolitica* strains would also affect only the bulkiness of the side chain because both are nonpolar aliphatic amino acids. As arginine is a positively charged amino acid, while glutamine is a polar uncharged amino acid, substitution of R69N in *Y. enterocolitica* strains may also affect enzyme activity. Lysine is a positively charged amino acid while threonine is a polar uncharged amino acid; substitution K98T is expected to affect the side chain of the protein. Asparagine is a polar

| blaA variant | Estimated Inhibition Constant, Ki | Free Energy of Binding (kcal/mol) | Interacting Residues |
|--------------|----------------------------------|----------------------------------|---------------------|
| blaA1        | 812.13 uM                        | −4.22                            | Hbonds 75S, 135S, 242S |
|              |                                  |                                  | Hydrophobic interactions 78K, 110Y, 137N, 221T, 239K, 240T, 241G, 249N |
| blaA2        | 649.90 uM                        | −4.35                            | Hbonds 75S, 78K, 135S |
|              |                                  |                                  | Hydrophobic interactions 110Y, 137N, 175N, 240T, 241G, 242S |
| blaA3        | 392.72 uM                        | −4.65                            | Hbonds 75S, 135S, 221T, 239K, 242S |
|              |                                  |                                  | Hydrophobic interactions 78K, 110Y, 134Y, 137N, 240T, 241G |
| blaA4        | 1.11 uM                          | −4.03                            | Hbonds 75S, 135S, 137N |
|              |                                  |                                  | Hydrophobic interactions 74C, 109S, 110Y, 171E, 175N, 242S |
| blaA5        | 159.42 uM                        | −4.98                            | Hbonds 135S, 240T, 242S, 239K |
|              |                                  |                                  | Hydrophobic interactions 75S, 221T, 241G, 248T, 249N, 277P, 279R, 282L |
| blaA6        | 150.09 uM                        | −5.18                            | Hbonds 135S, 240T, 242S |
|              |                                  |                                  | Hydrophobic interactions 75S, 221T, 239K, 241G, 248T, 249N, 277P, 279R, 282L |

Figure 4 | Molecular docking analysis of the six blaA variants with amoxicillin and clavulanic acid. Molecular interactions of docked blaA1 (a & b), blaA2 (c & d), blaA3 (e & f), blaA4 (g & h), blaA5 (i & j), blaA5 (k & l) and blaA6 (m & n) with amoxicillin and clavulanic acid respectively. The two antibiotics are represented by stick and the interacting amino acids are shown in dark color.
uncharged amino acid, while aspartate is a negatively charged amino acid; substitution of N101D in Y. enterocolitica strain 19 may influence enzyme activity due to charge in the properties of the side chain. Substitution of H111V and T225S in Y. enterocolitica strain E4 and L144V in Y. enterocolitica strains E2 and E4 would also affect only the bulkiness of the side chain of the protein without any profound effect on enzyme activity because of the similarity in the chemical nature of the substituent and the substrate. For example isoleucine and valine are nonaliphatic amino acids, threonine and serine are polar amino acids and leucine and valine both nonpolar amino acids.

Phylogenetic analysis based on amino acid sequences of blaA clustered the biotype 1A strains into 3 major groups. In our earlier study also the Y. enterocolitica biotype 1A strains clustered into three groups based on the profiles generated by the restriction analysis of blaA.

The analysis of amino acid sequences of blaA of Y. enterocolitica biotype 1A strains showed that although none of the substitution/mutation occurred in the four major motifs conserved across class A β-lactamases, or in the omega-loop region of blaA, yet there were differences in the specific activities of blaA. Amino acid substitutions which do not occur in the active site of the enzyme may create diverse local changes in the 3D structure of the enzyme and increase its conformational flexibility which may in turn affect the binding affinity with the substrate. To evaluate the role of such amino acid substitutions on binding affinity we classified the blaA of biotype 1A strains of Y. enterocolitica into six variants based on the mutation/substitution in the amino acid sequence. Three dimensional modeling of the blaA variants was carried out to correlate structural changes with the higher enzyme specific activities and MIC of different strains. In-silico docking of the blaA variants was carried out with amoxicillin and clavulanic acid to analyze if amino acid substitutions affected the residues involved in hydrogen bonding and hydrophobic interactions in the enzyme-ligand complex. It is well known that hydrogen bonds and hydrophobic interactions not only play a crucial role in molecular recognition and the overall stability of the protein structure; these also stabilize the protein-ligand complexes. Amoxicillin and clavulanic acid were chosen for docking experiments as both are penicillins and blaA is a penicillinase. Intermolecular hydrogen bonds formed between the modeled structures of blaA variants and amoxicillin/clavulanic acid were determined. Pymol analysis revealed that each blaA variant had its own drug-interacting active-site amino acids. In all blaA variants S-135, A-137 and S-242 participated in hydrogen bonding with amoxicillin, and S-135 in hydrogen bonding with clavulanic acid. Kumar et al. reported that the number of the residues involved in hydrogen bond interactions was an indicator of binding affinity of the enzyme with the ligand which might be decreased in mutant forms of class A β-lactamase. However, in our study no co-relation was observed between the number of residues involved in hydrogen bond interactions in blaA variants and their MIC for amoxicillin or clavulanic acid. Yi et al. also observed that the cetadizidine MIC and the relative frequency of substitutions in PenA, a class A β–lactamase of Burkholderia thailandensis did not co-relate well. It may be worthwhile to mention that the Y. enterocolitica isolates used in the present work were isolated from environmental as well as clinical settings. Variations/mutations associated with their respective blaAs might have evolved over time either in nature or in clinical settings in patients treated with antibiotics. Salverda et al. have also reported that most of the mutations that evolved in β-lactamases in clinical settings do not clearly co-relate with the stability or enhanced hydrolytic capability of the enzyme.

Variation in amino acid sequence is not the only factor that might contribute to differences in specific activities of β-lactamases. Enzyme activity may also be affected at the level of translation. In this regard, the secondary structure of mRNA plays an important role. When the secondary structures of mRNA of blaA variants were predicted using the web server mfold it was found that except for blaA4, all variants formed branched secondary structures. The mRNA secondary structure of blaA2 showed extensive branching and a lesser region of double stranded RNA helix compared to other blaA variants. On the other hand the mRNA secondary structure of blaA4 comprised mainly of double-stranded RNA helix of stacked base pairs with little branching. Several studies have shown that longer and stronger paired zones in mRNA secondary structure tend to be more stable, preventing the ribosome from breaking the pairing and helping translation to proceed. A lower translation rate means lesser number of copies of protein and hence a lower enzyme specific activity. Thus the secondary structures attained by mRNA of blaA2 and blaA4 might be the probable reasons for the higher and lower specific activities of their respective β-lactamases.

Overall the present work revealed that variations in promoter regions were not responsible for differential expression of blaA in biotype 1A strains of Y. enterocolitica. Analysis of amino acid sequences revealed that six types of blaA variants were present among biotype 1A strains of Y. enterocolitica. In-silico analysis of the blaA variants showed that amino acid substitutions did not contribute to the differential expression of the enzyme activities either. Rather the secondary structures attained by mRNA of blaA might underlie the differential expression of blaA in Y. enterocolitica. Further studies on secondary structures of mRNA of blaA gene will be helpful in better
strains of European origin were provided by J. Heesemann (Max von Pattenkofer origin were obtained from E. Carniel (Yersinia National Reference Laboratory and WHO Collaborating Center, Pasteur Institute, Paris, France). All these strains have also been deposited with the National repository i.e. Microbial Type Culture Collection (MTCC) and Gene Bank located at Institute of Microbial Technology, Chandigarh. Ten strains of *Y. enterocolitica* biotype 1A belonging to European and American origin were obtained from E. Carniel (Yersinia National Reference Laboratory and WHO Collaborating Center, Pasteur Institute, Paris, France). Another six biotype 1A strains of European origin were provided by J. Heesemann (Max von Pattenkofer Institute, Munich, Germany). The serovars, source of isolation, country of origin and reference laboratory accession numbers of these strains have been reported previously[20]. All strains were maintained on trypticase soy agar at 4°C.

**Determination of the Minimal Inhibitory Concentration (MIC)**, MIC of amoxicillin, amoxicillin-clavulanate, cefotaxime and cefpodoxime was determined for 25 strains of *Y. enterocolitica* biotype 1A using E-test (bioMerieux Inc., MO, USA) following the manufacturer’s instructions. Briefly, each strain was grown on Muller-Hinton agar plates at 25°C for 18–24 h. Single colonies from the agar plates were suspended in 1 ml of normal saline until a turbidity of 0.5 McFarland standard was reached (equivalent to ca. 1.2–10^8 CFU/ml). Sterile glass spreader was used to spread 100 μl of the cell suspension on the surface of Muller-Hinton agar plates. The E-test strips were placed on the surface of inoculated Muller-Hinton agar plates and incubated at 28°C for 16–18 h. The MIC values were recorded as the minimum concentration of the antibiotic (μg/ml) where no visible growth of the test organism was observed and were interpreted according to the guidelines of Clinical and Laboratory Standards Institute[21].

**Spectrophotometric assay of β-lactamases**. Thirty-five strains of *Y. enterocolitica* biotype 1A were used for assaying β-lactamase activity as reported by Stock and Weidmann[18] with slight modification. A loopful of bacterial growth was inoculated in 2 ml of trypticase soy broth at 28°C for 24 h. After the bacteria were sedimented by centrifugation at 4°C, washed thrice with chilled phosphate buffer (0.05 M, pH 7.0) and finally resuspended (1 g wet weight) in 5 ml of sonication buffer. The cells were disrupted by sonication on ice with three pulses of 30 sec each. The clear cell lysate, prepared by centrifugation at 12,000 g for 30 min at 4°C was used to assay the β-lactamase activity. The protein content of the lysates was estimated by Bradford’s method[22]. β-lactamase activity was assessed spectrophotometrically by hydrolysis of nitrocefin. The assay mixture contained 110 μl nitrocefin (0.05 μg/μl), 30 μg of crude cell lysates containing β-lactamase in a final volume of 1.0 ml of 50 mM phosphate buffer (pH 7.0). β-lactamase activity was monitored by measuring absorbance in the optical density at 486 nm for 10 min at 28°C. The specific activity of the enzyme was expressed as μmol of nitrocefin hydrolyzed/min/mg of protein and was calculated based on the molar extinction coefficient of 20,500 M⁻¹ cm⁻¹ for nitrocefin[23].

**Preparation of genomic DNA**. Each strain was grown overnight in trypticase soy broth at 28°C. One ml of the culture was pelleted at 8,000 × g for 10 min. The genomic DNA was prepared from each strain by using DNAeasy Tissue kit (Qiagen, Hilden, Germany) with modifications for Gram-negative bacteria according to the manufacturer’s recommendations. Purified DNA was eluted in sterile water and quantitated spectrophotometrically at 260 nm.

**PCR amplification of blaA** gene including the promoter and downstream region. For amplification of partial coding sequence of *blaA* including its promoter, primers A11f 5’CAAATCGCCAGCAGAACAGA 3’ and A12r 5’GGAGCGGTAAAGAAA3’ were designed for the predicted sequence of *blaA* along with the 350 bp upstream and downstream of the gene. The primers were synthesized from Microsynth GmbH (Balgach, Germany).

PCR amplification of the promoter region and partial coding sequence of *blaA* was carried out for 81 strains of *Y. enterocolitica* in a Thermal Cycler (MyCyclerTM, Bio-Rad, CA, USA). The PCR reaction mixture comprised of 1X PCR buffer (10 mM Tris-HCL, 1.5 mM MgCl₂, 1.5 mM KCl and 0.1% Triton X-100), 200 μM of each of the four dNTPs (MBI Fermentas GmbH, Germany), 10 pmol each of forward and reverse primers, 2 U of Taq DNA polymerase (NewEngland BioLabs, MA, USA) and 50–100 ng of genomic DNA in a total volume of 25 μl. For PCR, the initial denaturation was performed at 95°C for 5 min followed by 30 cycles of 30 sec at 95°C, 30 sec at 56°C and 90 sec at 72°C and a final extension for 10 min at 72°C. The PCR products were analyzed by electrophoresis on 1% (w/v) agarose gel. The gels were stained with ethidium bromide (0.5 μg/ml) and visualized under UV transilluminator.

**Phylogenetic analysis of blaA of Y. enterocolitica strains**. The amino acid sequences of *blaA* of 35 strains of *Y. enterocolitica* biotype 1A were also analyzed from evolutionary perspectives using the software MEGAS2.2 (Molecular Evolutionary Genetic Analysis). The evolutionary history was inferred using the Maximum Likelihood method based on the JTT matrix-based model (www.megasoftware.net).

**Selection of *Y. enterocolitica* strains for modeling of *blaA***. To investigate the role of point substitutions in amino acid sequence in differential expression of *blaA*, analysis of protein models of *blaA* variants was carried out. After analysis by Clustal, amino acid alignments of *blaA* of *Y. enterocolitica* strains representing type/(s) of *blaA* substitution(s) were selected for modeling of their β-lactamases. The *blaA* of Y. enterocolitica strains 8, 19, 20, 29, E2 and E4 were selected for modeling and accordingly named as *blaA1, blaA2, blaA3, blaA4, blaA5 and blaA6*.

**Homology modeling of β-lactamases***. Since the three dimensional (3D) structure of *Y. enterocolitica* is not known, the 3D structure of *blaA* variants *blaA1, blaA2, blaA3, blaA4, blaA5 and blaA6* were predicted by homology modeling. The pairwise alignment between the target and template sequences was performed with PDB- BLAST. Three-dimensional structures of *blaA1, blaA2, blaA3, blaA4, blaA5 and blaA6* were built using the software MODELLER 9.12 (http://salilab.org/ modeller/). Twenty models satisfying structural and sequence-template alignment were built for each protein, of which the 3D model with the lowest modeler objective function was selected. Following the modeling process, the structure was validated by PROCHECK and VERIFY3D. The PROCHECK program performs a detailed analysis of the stereochemistry of the modeled protein structure in the form of a Ramachandran plot by plotting the phi psi torsion angles. Population of phi psi torsion angles in the plot gives an assessment of the overall quality of the modeled structure and also highlights regions that may need further investigation[24]. The quality of the three-dimensional models was also assessed by VERIFY3D, which tests the compatibility of a protein structure with its amino acid sequence. For high-resolution, experimentally determined structures, the VERIFY3D scores are positive and consistently high i.e > 0.25.

**Molecular docking**. Molecular docking analysis of the modeled protein structures was carried out to identify if substitutions in the amino acid sequences of *blaA* resulted in variations in their binding affinity to β-lactam antibiotic amoxicillin and the β-lactamase inhibitor clavulanic acid. The structures of amoxicillin and clavulanic acid were retrieved from the PubChem compound database (http://pubchem.ncbi.nlm.nih.gov/). Subsequently the downloaded SDF file was converted to PDB format using iLab. Protein structures were pre-processed for docking using latest AutoDockTools (ADT) version by adding polar hydrogen atoms, removing all non-protein molecules from the modeled structure, adding Kolimam charges to the protein and converting it to PDBQT format. Ligand structures were also prepared using ADT, using the default method for preparing ligands for docking that added hydrogen and charges. The default rotatable bonds were accepted as well, and the structure was converted to PDBQT format. The search space for docking was determined visually by centering the Grid Box to the known binding site of the ligand and expanding the dimensions of the cubic search space to completely encompass the site. Docking was performed using AutoDock Vina (http://vina.scripps.edu/index.html) with default parameter settings other than the search space specification described above, and the mean predicted binding affinity from the set of predicted binding poses was accepted as the true binding affinity for the best docking run. The maximum number of poses per ligand was set to 10. The predicted binding of the ligand was compared to the known binding sites of β-lactamase in order to make predictions about differential responses with respect to each of the drugs. Interaction between ligand and target protein was analyzed by PyMOL[25].

**Analysis of mRNA secondary structure**. The secondary structure of mRNA of *blaA* variants was predicted using the mfold webservice at default parameters (http://mfold. rna.albany.edu/). The topologically optimal secondary structure of an RNA molecule taking into account many realistic physical parameters that affect the RNA folding, such as pH, temperature and local biases of RNA.

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**Author contributions**

Conceived and designed the experiments: N.S. and J.S.V. Bioinformatic analysis: M.K. Wrote the paper: N.S., J.S.V. and M.K.

**Additional information**

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