Characteristics of $\beta$-lactamases and their genes ($blaA$ and $blaB$) in Yersinia intermedia and Y. frederiksenii

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Abstract

Background: The presence of $\beta$-lactamases in Y. enterocolitica has been reported to vary with serovars, biovars and geographical origin of the isolates. An understanding of the $\beta$-lactamases in other related species is important for an overall perception of antibiotic resistance in yersiniae. The objective of this work was to study the characteristics of $\beta$-lactamases and their genes in strains of Y. intermedia and Y. frederiksenii, isolated from clinical and non-clinical sources in India.

Results: The enzymes, Bla-A (a constitutive class A penicillinase) and Bla-B (an inducible class C cephalosporinase) were found to be present in all the clinical and non-clinical strains of Y. intermedia and Y. frederiksenii by double disc diffusion method. The results showed differential expression of Bla-A as indicated by presence/absence of synergy whereas expression of Bla-B was quite consistent. The presence of these enzymes was also reflected in the high minimum inhibitory concentrations, MIC$_{50}$ (126–1024 mg/L) and MIC$_{90}$ (256–1024 mg/L) of $\beta$-lactam antibiotics against these species. Restriction fragment length polymorphism (RFLP) revealed heterogeneity in both $blaA$ and $blaB$ genes of Y. intermedia and Y. frederiksenii. The $blaA$ gene of Y. intermedia shared significant sequence identity (87–96%) with $blaA$ of Y. enterocolitica biovars 1A, 1B and 4. The sequence identity of $blaA$ of Y. frederiksenii with these biovars was 77–79%. The sequence identity of $blaB$ gene of Y. intermedia and Y. frederiksenii was more (85%) with that of Y. enterocolitica biovars 1A, 1B and 2 compared to other species viz., Y. bercovieri, Y. aldovae and Y. ruckeri. Isoelectric focusing data further revealed that both Y. intermedia and Y. frederiksenii produced Bla-A (pI 8.7) and "Bla-B like" (pI 5.5–7.1) enzymes.

Conclusion: Both Y. intermedia and Y. frederiksenii showed presence of $blaA$ and $blaB$ genes and unequivocal expression of the two $\beta$-lactamases. Limited heterogeneity was detected in $blaA$ and $blaB$ genes as judged by PCR-RFLP. Phylogenetic relationships showed that the two species shared a high degree of identity in their $bla$ genes. This is the first study reporting characteristics of $\beta$-lactamases and their genes in strains of Y. intermedia and Y. frederiksenii isolated from Asian region.
mented human pathogens. *Y. pestis* is the etiologic agent of plague (black death) while the other two are known to cause a variety of gastrointestinal syndromes [2]. The remaining eight species namely *Y. intermedia*, *Y. frederiksenii*, *Y. kristensenii*, *Y. bercoveroi*, *Y. mollaretii*, *Y. aldovae*, *Y. rohdei* and *Y. ruckeri* have generally been termed as “*Y. enterocolitica*-like” species though each is a distinct species. Although most commonly isolated from sources like fresh water and food, these have only been infrequently recovered from human clinical specimens [1]. But, some of these, especially *Y. intermedia* and *Y. frederiksenii* may constitute as much as 18–32% of all *Yersinia* isolated from stools of diarrheic patients [3]. However, not much information is available about these, and Sulakvelidze termed them as the ignored species [1]. Nevertheless, *Y. intermedia* [4], *Y. frederiksenii* [5], *Y. bercoveroi* [6] and *Y. kristensenii* [7] have been strongly implicated in cases of diarrhoea. It is therefore important to know more about these species to understand their public health significance.

The production of β-lactamases is an important mechanism of resistance to β-lactam antibiotics. In *Y. enterocolitica*, the distribution and production of β-lactamases namely Bla-A (a constitutive class A enzyme) and Bla-B (an inducible class C cephalosporinase or AmpC) has been studied widely and reported to vary with biovars and geographical origin of the strains [8-11]. However, not much is known about the β-lactamases of other important species like *Y. intermedia*, *Y. frederiksenii* and *Y. kristensenii*. Recently, *Y. intermedia*, *Y. frederiksenii*, and some strains of *Y. kristensenii* and *Y. rohdei* were reported to produce two β-lactamases [12,13]. On the other hand, *Y. bercoveroi* and *Y. mollaretii* have been reported to produce only AmpC β-lactamase, while *Y. aldovae* and *Y. ruckeri* showed either low levels of AmpC or no expression of β-lactamases at all [14]. This information has been inferred from MIC data of β-lactam antibiotics. Schiefer et al [15] recently characterized β-lactamases of *Y. Frederikenii*, *Y. Bercoveroi*, *Y. Aldovae* and *Y. Ruckeri* and reported that except for *Y. Frederikenii*, all expressed only an AmpC β-lactamase. Mammeri et al [16] cloned and sequenced the complete ampC gene of *Y. Ruckeri* and found that it shared low level of identity with the known chromosomal and plasmid AmpC enzymes of closely related members, viz., *Enterobacter cloacae*, *E. aerogenes* and *Citrobacter freundii*. Much of these data however pertains to strains isolated in Europe. It would be worthwhile to study the β-lactamases and bla genes of strains isolated from other parts of the world to understand the global drug resistance of yersinia. No data is available on β-lactamases of "*Y. enterocolitica*-like" species isolated from Asian region. Therefore, the objective of this work was to detect and characterize the β-lactamases and bla genes of *Y. intermedia* and *Y. Frederikenii* isolated from clinical and non-clinical sources in India.

**Results and discussion**

**Phenotypic detection of Bla-A and Bla-B enzymes**

All the clinical and non-clinical isolates of *Y. intermedia* and *Y. Frederikenii* showed presence of Bla-A as indicated by the appearance of a small zone of inhibition of 2–8 mm radii (10–22 mm diameter) around ticarcillin 75 μg disc (Table 1). However, synergy, the appearance of a characteristic additional zone of inhibition between ticarcillin and the adjacent clavulanate disc, was detected in only 26% (9/34) of the isolates of *Y. intermedia* and one isolate of *Y. Frederikenii*. In an earlier study, similar observations regarding synergy were made for strains of *Y. enterocolitica* biovar 1A [8]. In the present study, Bla-B, the inducible cephalosporinase, was detected unequivocally based on the characteristic flattening of inhibition zone around cefotaxime disc adjacent to the imipenem disc in all the strains (Table 1). Tzelepi et al [17] also reported detection of a broad spectrum penicillinase (Bla-A) and an inducible cephalosporinase (Bla-B) in all the thirty aquatic isolates of *Y. intermedia*. Our study extends this information further to indicate that Bla-A was also present in *Y. intermedia* isolated from clinical and non-clinical (pig/wastewater) sources. In addition, the present study also showed unequivocal presence of Bla-A and Bla-B in strains of *Y. Frederikenii*, which was inferred earlier on the basis of MIC data only [12,13].

For Bla-A, a larger zone of inhibition (4–8 mm) around ticarcillin disc (75 μg) was observed for wastewater isolates of *Y. intermedia* compared to that of clinical (1.5–2.0 mm) isolates. The zone of inhibition (1.5–8 mm) around *Y. intermedia* was nevertheless larger compared to that around the strains of *Y. Frederikenii* (1.5–3.6 mm). Also, synergy i.e., appearance of an additional zone of inhibition around ticarcillin disc, was observed in the clinical isolates only. As these phenomena are highly dependent on the degree of the expression of enzyme, these observations suggested differential expression of Bla-A as reported earlier for strains of *Y. enterocolitica* biovar 1A [8].

**Minimum Inhibitory Concentration (MIC)**

All the strains were resistant to penicillins and cephalosporins tested. When analyzed separately, no difference was observed in the antibiotic susceptibilities of *Y. intermedia* and *Y. Frederikenii*. Thus, the combined results are shown in Table 2. The MIC<sub>50</sub> of amoxicillin was 512 mg/L for the clinical and 1024 mg/L for the non-clinical strains, whereas MIC<sub>90</sub> was 1024 mg/L and 2048 mg/L, respectively. For co-amoxiclav, the MIC<sub>50</sub> ranged from 128–256 mg/L and MIC<sub>90</sub> was 256–512 mg/L for both clinical and non-clinical strains. The strains were equally resistant to cephalosporins. Amongst these, minimum resistance (MIC<sub>50</sub> 32–64 mg/L and MIC<sub>90</sub> 128 mg/L) was seen against cefotaxime for strains of *Y. intermedia* and *Y. Frederikenii*. For ceftazidime, the MIC<sub>50</sub> and MIC<sub>90</sub> were
512 mg/L and 1024 mg/L, respectively, and for cefepime, the values ranged from 256–1024 mg/L. Contrary to these, relatively lower MICs of penicillins and cephalosporins have been reported for *Y. intermedia* and *Y. frederiksenii* isolated in other parts of the world [13,17,18]. This may be due to difference in the sources from which organisms were isolated or their geographical origin. Interestingly, Tzelepi et al [17] found that the strains of *Y. intermedia* isolated in Europe were sensitive to both cefotaxime and ceftazidime. The antibiotic resistance data of strains of *Y. intermedia* and *Y. frederiksenii* was in accordance with the preliminary reports from our laboratory earlier [19].

**PCR amplification and restriction digestion of blaA gene**

The β-lactamase genes namely the blaA and blaB were detected by PCR amplification and found to be present in all strains of *Y. intermedia* and *Y. frederiksenii*.

Initially, an attempt was made to amplify blaA using published primers (A9-f and A10-r) of *Y. enteroocolitica* biovar 1A [20]. However, though some of the strains of *Y. intermedia* yielded expected amplicon, none of the *Y. frederiksenii* strains gave any amplicon. This may be attributed to differences in the gene sequences of blaA of *Y. intermedia* and *Y. frederiksenii* compared to that of *Y. enteroocolitica* biovar 1A. Thus, to amplify blaA of *Y. intermedia* and *Y. frederiksenii*, new internal primers (A7-f and A8-r) were designed. When these primers were used for amplification of blaA, the expected 450 bp amplicon was obtained for all the forty-nine strains of *Y. intermedia* and *Y. frederiksenii*.

Restriction digestion of blaA with NciI revealed that a single site for NciI was present in blaA of only a few strains of *Y. intermedia* and *Y. frederiksenii* as either an uncut DNA or two fragments of 350 bp and 100 bp were obtained (Fig. 1). However, restriction with HaeIII gave three types of patterns having molecular weights 210, 190 and 60 bp (pattern I), 210, 190 and 55 bp (pattern II), and 350 and 100 bp (pattern III), for both *Y. intermedia* and *Y. frederiksenii*. The phylogenetic relationship and genetic heterogeneity in blaA was studied by constructing a concatenated dendrogram (Fig. 2) of the NciI and HaeIII restriction profiles. The strains of *Y. intermedia* grouped into three major clusters: A, B and C. Cluster A was formed by three wastewater isolates (T/Y/65, R/Y/59 and O/Y/60, all of serovar O:40). Cluster B was most heterogenous comprising clinical, wastewater and pig throat isolates while the cluster C was represented predominantly by pig throat isolates (Fig. 2a). The clusters B and C were divided further into two subclusters each. The blaA-RFLP grouped strains of *Y. frederiksenii* into two major clusters – A and B (Fig. 2b). Except for one, all the strains of clinical origin belonged to cluster B. As observed for *Y. intermedia*, the strains of *Y. frederiksenii* isolated from pig throat also clustered separately into subgroup BII (Fig. 2b). Earlier, work from our laboratory reported that some clinical and non-clinical strains of *Y. enteroocolitica* biovar 1A formed separate clusters based on blaA-RFLP [20].

Table 1: Detection of β-lactamases Bla-A and Bla-B in *Y. intermedia* and *Y. frederiksenii* isolated from India

| Species         | Source (n) | Detection of Bla-A | Detection of Bla-B |
|-----------------|------------|--------------------|--------------------|
|                 |            | Number positive (%) | Synergy (%)        | Number positive (%) |
| *Y. intermedia* | Clinical (4) | 4 (100)            | -                  | 4 (100)            |
|                 | Wastewater (8) | 8 (100)            | 4 (50)             | 8 (100)            |
|                 | Pig throat (22) | 22 (100)           | 5 (22.7)           | 22 (100)           |
| *Y. frederiksenii* | Clinical (6) | 6 (100)            | -                  | 6 (100)            |
|                 | Pig throat (9) | 9 (100)            | 1 (11.1)           | 9 (100)            |

n number of strains
1 number of strains with annular radii between 2–8 mm (diameter 10–22 mm) of the zone of inhibition around ticarcillin disc
2 Synergy – additional zone of inhibition between ticarcillin and co-amoxiclav discs
3 number of strains that showed characteristic flattening of the zone of inhibition around cefotaxime disc.

Table 2: Susceptibility of *Y. intermedia* and *Y. frederiksenii* to selected antibiotics

| Source (n) | AMX  | AMC  | CTX  | CAZ  | FEP  | AMX  | AMC  | CTX  | CAZ  | FEP  |
|------------|------|------|------|------|------|------|------|------|------|------|
| Clinical (10) | 512  | 128  | 64   | 512  | 512  | 1024 | 256  | 1024 | 1024 |
| Non-clinical (39)* | 1024 | 256  | 32   | 512  | 256  | 2048 | 512  | 128  | 1024 |

n Number of strains; AMX, Amoxicillin; AMC, Co-amoxiclav; CTX, Cefotaxime; CAZ, Ceftazidime; FEP, Cefepime
*Wastewater (8 strains) and Pig throat (31 strains)
clearly revealed heterogeneity in was observed in this study. The present work however, PCR amplification of PCR amplification and restriction digestion of (HaeIII and RsaI) were used, no heterogeneity was discerned in blaB gene of Y. enterocolitica biovar 1A [20]. This suggested that blaB gene of Y. intermedia and Y. frederiksenii was more heterogenous compared to that of Y. enterocolitica.

Sequencing of bla genes
The blaA gene sequence of Y. intermedia showed high degree of identity with Y. enterocolitica biovar 1A (96.8%) [20], Y. enterocolitica 8081 biovar 1B (90%) and Y. enterocolitica Y-56 biovar 4 (87.7%) [25]. The identity of blaA of Y. frederiksenii with that of the above-mentioned organisms was however found to be 77%, 79.5% and 79.8% respectively, suggesting a distinct lineage of Y. frederiksenii. The sequence identity of blaA gene of Y. intermedia and Y. frederiksenii with other members of the family Enterobacteriaceae namely Klebsiella oxytoca and Citrobacter koseri was comparatively very low and ranged from 48–50% for Y. intermedia and 62–63% for Y. frederiksenii. Furthermore, identity of blaA of Y. intermedia and Y. frederiksenii with Burkholderia cepacia, a non-enterobacterial species was 47.7% and 60%, respectively.

The deduced amino acid sequences of Bla-A of Y. intermedia and Y. frederiksenii were analyzed to check similarity in β-lactamases at protein level (Fig. 4). The SXXK tetrad, characteristic of β-lactamases possessing a serine active site, was present at position 70–73 [26]. In addition, two structural motifs characteristic of class A β-lactamases, were also found to be present in Bla-A of Y. intermedia and Y. frederiksenii: SDN at position 130–132 and KTG at position 234–236 along with the motif responsible for omega (Ω) loop formation, i.e., 166(EPDLN)170. The amino acid sequence alignment revealed high degree of identity (87–94%) of Bla-A of Y. intermedia and Y. frederiksenii with that of Y. enterocolitica biovars 1A, 1B and 4. The percent identity of Bla-A of Y. frederiksenii with K. oxytoca and C. koseri was found to be higher (69–71%) compared to that of Y. intermedia (49–53%). Overall, the data suggested that class A β-lactamase of Y. intermedia shared high identity with Y. enterocolitica, and that of Y. frederiksenii with other members of the family Enterobacteriaceae and B. cepacia. This seems to reiterate the distinct lineage of Y. frederiksenii as suggested by other investigators based on 16S rDNA and gyrase B genes sequence analyses [27].

The nucleotide sequences of blaB of Y. intermedia and Y. frederiksenii were found to have 85% identity with that of

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Several reports indicate that genetic background of antibiotic resistance genes influence antibiotic susceptibility profiles [21,22]. However, no such unequivocal relationship between blaA-RFLP type and antibiotic susceptibility was observed in this study. The present work however, clearly revealed heterogeneity in blaA gene of Y. intermedia and Y. frederiksenii, which may account for differential expression of Bla-A enzyme as seen in double disc diffusion method. Nevertheless, the heterogeneity was only limited as observed earlier for blaA gene of Y. enterocolitica biovar 1A strains [20]. In Moraxella catarrhalis, the two class A β-lactamase genes namely BRO-1 and BRO-2, have been reported to give different restriction profiles with BclI [23,24].

**PCR amplification and restriction digestion of blaB gene**

PCR amplification of blaB gene resulted in 850 bp product for all the forty-nine strains of Y. intermedia and Y. frederiksenii. The restriction digestion of blaB of Y. intermedia and Y. frederiksenii with HaeIII gave two types of patterns i.e., 700 bp and 100 bp, and 600 bp and 120 bp (Fig. 1). With RsaI, except for the three wastewater isolates of Y. intermedia (T/Y/65, R/Y/59 and O/Y/60), all the clinical and majority of the wastewater isolates grouped together in cluster B, whereas group C consisted exclusively of pig throat isolates (84%) (Fig. 3a). Cluster analysis of restriction profiles of Y. frederiksenii revealed that except for two, all clinical and pig throat strains grouped together in one major cluster (Fig. 3b). In a previous study, when the same restriction enzymes (HaeIII and RsaI) were used, no heterogeneity was discerned in blaB gene of Y. enterocolitica biovar 1A [20]. This suggested that blaB gene of Y. intermedia and Y. frederiksenii was more heterogenous compared to that of Y. enterocolitica.
Figure 2
Phylogenetic analysis of blaA gene of (A) Y. intermedia and (B) Y. frederiksenii. NAG, non-agglutinable; -, Not determined; St, Serotype; Bt, Biotype.
Y. enterocolitica biovar 1A, Y. enterocolitica 8081 biovar 1B and Y. enterocolitica IP97 biovar 2, and 77% with Y. bercovieri. But it was only 50–55% with Y. aldovae and Y. ruckeri. The deduced amino acid sequence similarity of Bla-B of Y. intermedia and Y. frederiksenii with Y. enterocolitica biovars 1A, 1B and 2 ranged from 89–92%. The comparison of these sequences with that of other “Y. enterocolitica-like” species such as Y. bercovieri, Y. aldovae and Y. ruckeri showed percent identity of 80%, 53% and 49% respectively. Thus, the phylogenetic relationships as discerned by multilocus enzyme electrophoresis [28,29] were reiterated in antibiotic resistance genes, both at the nucleotide and the protein level. The deduced amino acid sequence of Bla-B also revealed presence of the three characteristic motifs viz., 64SXXS67, 150YAN152 and 314KTG316 (Fig. 5).

Molecular weight determination of Bla-A and Bla-B
Molecular weight determination on SDS-PAGE showed two distinct bands of 37 kDa and 29 kDa for all strains of Y. intermedia and Y. frederiksenii. The band at 37 kDa was characteristic of Bla-A enzyme, as the molecular weight of most class A β-lactamases from other organisms namely Y. enterocolitica [20], Burkholderia pseudomallei [30] and Citrobacter sedlaki [31] has been reported to vary between 29–35 kDa. The identity of this band was also confirmed by specific inhibition with clavulanic acid. The band at 29 kDa corresponded to Bla-B (AmpC) as indicated by its specific inhibition with aztreonam. The molecular weight of inducible cephalosporinases or AmpC of other species namely Y. enterocolitica [32], Y. aldovae, Y. bercovieri, Y. ruckeri and Y. frederiksenii [15], and Serratia marcescens [33], has however been reported to be in the range of 34–40 kDa. The 29 kDa as found in the present study was similar to 29 kDa AmpC of Vibrio fischeri [34] and Y. enterocolitica biovar 1A [20]. Since most studies cited above used SDS-PAGE to determine the molecular weight, the differences cannot be attributed to methodology.

Isoelectric focusing analysis of Bla-A and Bla-B
Isoelectric focusing of β-lactamases of fifteen strains (8 Y. intermedia and 7 Y. frederiksenii) revealed a single band in the alkaline region of the gel at pI 8.7 (Table 3) that corresponded to Bla-A as indicated by its inhibition by clavulanic acid. Tzelepi et al [35] reported that Bla-A of Y. intermedia focused at pl 9.0–9.5. The Bla-A of Y. enterocolitica with pl 8.7 has been reported by several investigators [20,36,37].

The inducible cephalosporinase or AmpC β-lactamase focused as multiple bands in the acidic region of the gel at various pl values. The identity of these bands was confirmed both by induction with imipenem that made the bands more prominent, and also by specific inhibition with aztreonam. Two major bands in acidic region of the gel at pl 6.5 and 6.8 were observed for the clinical strains of Y. intermedia. The AmpC of non-clinical strains of Y. intermedia focused at pl ranging from 5.5 to 7.1 (Table 3). Two additional bands (pl 7.8 and 8.0) were observed in the pig throat strains. The only report on Bla-B (AmpC) of Y. intermedia reported pl to be between 5.5 to 6.1 [35]. The Bla-B of all the strains of Y. frederiksenii also appeared as multiple bands at pl 6.0, 6.8 and 8.0. The strains of Y. enterocolitica, for which most information is available in literature [36,37], produced Bla-B with pl 5.3–5.7 except biovar 1A strains, the Bla-B of which focused at pl 6.8 and 7.1. Consequently, the Bla-B of biovar 1A strains has been termed as “Bla-B like” [11]. The present study indicated that, like biovar 1A strains, Y. intermedia and Y. frederiksenii too produced “Bla-B like” enzyme.

Nucleotide sequence accession numbers
The nucleotide sequence data of blaA and blaB has been submitted to NCBI GenBank under accession numbers [GenBank: DQ424965 and GenBank: DQ656113], and [GenBank: DQ424967 and GenBank: DQ424968] respectively.

Conclusion
The two β-lactamases namely Bla-A and Bla-B were detected in all clinical and non-clinical strains of Y. intermedia and Y. frederiksenii isolated from India. Differential expression of Bla-A but not Bla-B was observed by double disc diffusion method. Both Y. intermedia and Y. frederiksenii were highly resistant to β-lactam antibiotics. PCR-RFLP revealed that blaA and blaB genes of both Y. intermedia and Y. frederiksenii were heterogeneous. Phylogenetic relationships also showed that the two species shared high degree of identity in their bla genes. Isoelectric focusing data revealed that both Y. intermedia and Y. frederiksenii produced Bla-A and “Bla-B like” enzymes. This is the first study in which β-lactamases (Bla-A and Bla-B) and β-lactamase genes (blaA and blaB) of Y. intermedia and Y. frederiksenii isolated from Asian region have been investigated.

Methods
Bacterial strains
Thirty four strains of Y. intermedia and fifteen of Y. frederiksenii isolated in India [38,39] from different sources namely diarrheic human subjects (10 strains), pig throat (31 strains) and wastewater (8 strains) were examined. The strains were authenticated by, and have been deposited with the Yersinia National Reference Laboratory and WHO Collaborating Centre at Institut Pasteur (Paris), France. The details of the strains are given in Table 4.

Antibiotics and chemicals
Mueller-Hinton agar (MHA), tryptone glucose yeast extract (TGYE) agar, tryptone soya broth (TSB) and antibiotic discs, namely ticarcillin 75 μg and imipenem 10 μg
Figure 3
Phylogenetic analysis of blaB gene of (A) *Y. intermedia* and (B) *Y. frederiksenii*. NAG, non-agglutinable; -, Not determined; St, Serotype; Bt, Biotype.
Detection of the enzymes Bla-A and Bla-B was carried out by double disc diffusion tests as described previously by \cite{chemotherapy}.

The MICs of five selected antibiotics namely amoxicillin, co-amoxiclav, cefepime, ceftazidime and cefepime were determined in Mueller-Hinton broth by microbroth dilution technique using the methodology described by the Working Party of the British Society for Antimicrobial Chemotherapy \cite{}. 

Minimum Inhibitory Concentrations (MIC)

The MICs of five selected antibiotics namely amoxicillin, co-amoxiclav, cefepime, ceftazidime and cefepime were determined in Mueller-Hinton broth by microbroth dilution technique using the methodology described by the Working Party of the British Society for Antimicrobial Chemotherapy \cite{}. 

PCR amplification of blaA and blaB genes

Total genomic DNA from each strain was extracted by DNA extraction kit (Qiagen, Germany) as per the manufacturer's instructions. PCR amplification of blaA gene from all strains of \textit{Y. intermedia} and \textit{Y. frederiksenii} was performed using the primers, A7-f (5' TATGCCCCGATGCTTCAAGGTTGACGTTCAACAGCGGAGGACAGCAGTGGTGGGAGGCAAGCAGCAGCTGCGTCGCGTGGTGGGAATGCGGGGCTCAATCATTGGTGGT 3') and blaB (5' CTAAACATGCTGAAGACTTATCAGCAGCTGCGTCGCGTGGTGGGAATGCGGGGCTCAATCATTGGTGGT 3').
β-lactamases are underlined and in boldface. The conserved motifs (64SXK67, 150YSN152 and 314KTG316) typical of class C β-lactamase neighbors. Asterisks indicate identical amino acids. The con-

Figure 5

Amino acid sequence alignment of AmpC β-lactamase (Bla-B) of Y. intermedia (strain C-702) and Y. frederiksenii (strain C-115) and the nearest class C β-lactamase neighbors. Asterisks indicate identical amino acids. The conserved motifs (64SXK67, 150YSN152 and 314KTG316) typical of class C β-lactamases are underlined and in boldface. The GenBank accession numbers of the β-lactamases are as follows: Y.fred and Y.inter (ABD84040 and ABD84041, present study), Y.ent_1 (AAZ66331, Y. enterocolitica biovar 1A). Y.ent_2 (CAA44850, Y. enterocolitica, IP97 biovar 2), Y.ent_1B (Y. enterocolitica 8081 biovar 1B), Y.berc (AAO21212, Y. bercovieri), Y.ald (AAO2111, Y. aldovae) and Y.ruck (AAM94804, Y. ruckeri).

Table 3: Isoelectric points (pI) of β-lactamases (Bla-A and Bla-B) of Y. intermedia and Y. frederiksenii

| Strains | pI | Bla-A | Bla-B |
|---------|----|-------|-------|
| Y. intermedia |
| Clinical | 4 | 8.7 | 6.5, 6.8, 7.5 |
| Non-clinical | 4 | 8.7 | 6.0, 6.8, 7.1, 7.8, 8.0 |
| Y. frederiksenii |
| Clinical | 6 | 8.7 | 6.0, 6.8, 8.0 |
| Non-clinical | 3 | 8.7 | 6.0, 6.8, 8.0 |

n: number of strains
aWastewater (2 strains) and pig throat (2 strains)
bIsolates from pig throat
instructions. The digestion mixture was incubated at 37 °C for 8 hours and resolved on 2% agarose gel in 1 × TBE (Tris-Borate-EDTA). The gels were stained with ethidium bromide (0.5 μg/ml) and photographed under UV illumination. Similarity amongst blaA and blaB was estimated by cluster analysis of the restriction profiles of each gene using Jaccard's similarity coefficient and a UPGMA dendrogram was constructed separately for each gene using Diversity Database software (Bio-Rad, USA).

Table 4: Biotypes, serotypes and sources of strains of Y. intermedia and Y. frederiksenii used in this study

| S. No. | Strain No. | Species | Serotype | Biotype | Source  | Ref. Lab no. |
|--------|------------|---------|----------|---------|---------|--------------|
| 1      | K/Y/30     | Y. intermedia | O:40     | 4       | Wastewater | IP26142      |
| 2      | K/Y/31     | Y. intermedia | O:40     | 4       | Wastewater | IP26143      |
| 3      | T/Y/33     | Y. intermedia |         |         |          |              |
| 4      | O/Y/43     | Y. intermedia | O:40     | 4       | Wastewater | IP26146      |
| 5      | Ko/Y/57    | Y. intermedia | NAG      | 2       | Wastewater | IP26306      |
| 6      | R/Y/60     | Y. intermedia | O:40     | 4       | Wastewater | IP26309      |
| 7      | T/Y/65     | Y. intermedia | O:40     | 4       | Wastewater | IP26318      |
| 8      | C-594      | Y. intermedia |         |         |          |              |
| 9      | C-702      | Y. intermedia |         | 2       | Clinical   | IP27477      |
| 10     | C-714      | Y. intermedia |         | 7.8-8   | Clinical   | IP27478      |
| 11     | C-752      | Y. intermedia |         |         | Clinical   | IP27479      |
| 12     | C-752      | Y. intermedia |         |         | Clinical   | IP27479      |
| 13     | P-60       | Y. intermedia |         |         | Pig throat |              |
| 14     | P-114      | Y. intermedia |         |         | Pig throat |              |
| 15     | P-122      | Y. intermedia |         |         | Pig throat |              |
| 16     | P-144      | Y. intermedia |         |         | Pig throat |              |
| 17     | P-185      | Y. intermedia |         |         | Pig throat |              |
| 18     | P-225      | Y. intermedia |         |         | Pig throat |              |
| 19     | P-226      | Y. intermedia |         |         | Pig throat |              |
| 20     | P-262      | Y. intermedia |         |         | Pig throat |              |
| 21     | P-337      | Y. intermedia |         |         | Pig throat |              |
| 22     | P-360      | Y. intermedia |         |         | Pig throat |              |
| 23     | P-364      | Y. intermedia |         |         | Pig throat |              |
| 24     | P-366      | Y. intermedia |         |         | Pig throat |              |
| 25     | P-368      | Y. intermedia |         |         | Pig throat |              |
| 26     | P-369      | Y. intermedia |         |         | Pig throat |              |
| 27     | P-387      | Y. intermedia |         |         | Pig throat |              |
| 28     | P-396      | Y. intermedia |         |         | Pig throat |              |
| 29     | P-402      | Y. intermedia |         |         | Pig throat |              |
| 30     | P-430      | Y. intermedia |         |         | Pig throat |              |
| 31     | P-459      | Y. intermedia |         |         | Pig throat |              |
| 32     | P-460      | Y. intermedia |         |         | Pig throat |              |
| 33     | P-468      | Y. intermedia |         |         | Pig throat |              |
| 34     | P-474      | Y. intermedia |         |         | Pig throat |              |
| 35     | C-115      | Y. frederiksenii | NAG |         | Clinical | IP27388      |
| 36     | C-203      | Y. frederiksenii | 35 |         | Clinical | IP27389      |
| 37     | C-249      | Y. frederiksenii | 35 |         | Clinical | IP27390      |
| 38     | C-503      | Y. frederiksenii | 35 |         | Clinical | IP27399      |
| 39     | C-567      | Y. frederiksenii | 35 |         | Clinical | IP27401      |
| 40     | C-580      | Y. frederiksenii | 35 |         | Clinical | IP27402      |
| 41     | P-180      | Y. frederiksenii |         |         | Pig throat |              |
| 42     | P-236      | Y. frederiksenii |         |         | Pig throat |              |
| 43     | P-238      | Y. Frederiksenii |         |         | Pig throat |              |
| 44     | P-240      | Y. Frederiksenii |         |         | Pig throat |              |
| 45     | P-257      | Y. Frederiksenii |         |         | Pig throat |              |
| 46     | P-259      | Y. Frederiksenii |         |         | Pig throat |              |
| 47     | P-263      | Y. Frederiksenii |         |         | Pig throat |              |
| 48     | P-270      | Y. Frederiksenii |         |         | Pig throat |              |
| 49     | P-273      | Y. Frederiksenii |         |         | Pig throat |              |

* All strains were isolated from New Delhi, India
NAG: Non-agglutinable
IP: Yersinia National Reference Laboratory and WHO Collaborating Centre, Institut Pasteur, Paris (France)
Sequencing of blaA and blaB genes

The complete CDS of blaA gene (896 bp) of Y. intermedia was amplified with primers A9-f and A10-r [20]. However, CDS of blaA of Y. frederiksenii could not be amplified using these primers. Thus, partial sequence of blaA (450 bp) of Y. frederiksenii was amplified using primers A7-f and A8-r. The amplicons of blaA and blaB genes of representative strains, each of Y. intermedia and Y. frederiksenii, were purified using QIA Quick Gel Extraction Kit (Qiagen, Germany) and sequenced by Big Dye Terminator Cycle Sequencing Ready Reaction kit using ABI 310 Genetic Analyzer (Applied Biosystems, Germany). The sequences obtained were analyzed for homology with bla genes in the existing GenBank database using Blastn [43]. The deduced amino acid sequences of the proteins were aligned with class A (for Bla-A) and class C (for Bla-B) β-lactamases of other members of the family Enterobacteriaceae using ClustalW [44].

Molecular weight determinations of Bla-A and Bla-B enzymes

The molecular weights of the two β-lactamases were determined on SDS-PAGE. Crude cell lysate containing enzymes was prepared by sonication of washed cell pellet as described earlier [8]. For induction of enzyme Bla-B, imipenem (final concentration 0.5 mg/l) was added during the log phase of cell culture. The protein concentration was estimated by Lowry’s method [45]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli [46]. Briefly, 50 μg of protein was loaded on 12% resolving and 5% stacking SDS-polyacrylamide gel (Mini Protean III, Bio-Rad, USA) along with the medium range molecular weight protein marker (Bangalore Genei, India). The electrophoresis was carried out at 80 V for 2 hours. For renaturation, the gels were washed twice, for 45 min each, in renaturation buffer (100 mM Tris HCl, pH 7.0 and 0.1% Triton-X-100) under mild shaking. β-lactamase bands were visualized by overlaying the gels with 0.5 mg/ml nitrocefin for 2 min. The identity of the bands was further confirmed by specific inhibition with 40 μM clavulanic acid for Bla-A or 20 μM aztreonam for Bla-B.

Isoelectric focusing (IEF) of Bla-A and Bla-B enzymes

IEF of the crude enzyme extract (3 μg of protein) was performed in 6% polyacrylamide gel containing 2% ampholyte of pH 3 to 10 (Biolyte Ampholyte, Bio-Rad, USA) using Mini IEF cell (Bio-Rad, USA) according to the protocol specified by the manufacturer. A broad range IEF standard with pl ranging from 4.45–9.6 (Bio-Rad, USA) was used as pl marker. The β-lactamase bands were visualized by overlaying the IEF gel with nitrocefin (0.5 mg/ml). The identity of the bands was further confirmed by specific inhibition with clavulanic acid and aztreonam as described above.

Authors’ contributions

SM (first author) carried out the major part of the work namely PCR amplification of the genes, PCR-RFLP, MIC, IEF, analysis and interpretation of data, and drafted the manuscript. SM (second author) and SS carried out disc diffusion tests for detection of β-lactamases and participated in molecular weight determination and IEF. JSV conceived the study, coordinated and supervised the work and helped in drafting the final manuscript. All authors have read and approved the manuscript.

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