**Title: Molecular Insights into Antimicrobial Resistance Traits of Multidrug Resistant Enteric Pathogens isolated from India**

Pawan Kumar[1]*, Satyabrata Bag[1]*, Tarini Shankar Ghosh[1]*, Prasanta Dey[1]*, Mayanka Dayal[1], Bipasa Saha[1], Jyoti Verma[1], Archana Pant[1,2], Shruti Saxena[1], Anbumani Desigamani[1], Preety Rana[1], Dhirendra Kumar[3], Naresh C Sharma[3], Pranita Hanpude[4], Tushar K Maiti[4], Asish K Mukhopadhyay[5], Rupak K Bhadra[6], G Balakrish Nair[1,7], Thandavarayan Ramamurthy[1], Bhabatosh Das[1,3@]

## Supplementary information S1

### 1. Bacterial species isolated and used for antibiotic susceptibility test in this study

*Aeromonas spp, Escherichia coli, Klebsiella pneumonia, Providencia stuartii, Pseudomonas aeruginosa, Salmonella enterica* Group (*AbonyII, Agona, Bareilly, Bovismorbificans, Denver, Emek, Enteritidis, Give, Hiduddify, Hindmarsh, Idikan, Infantis, Kentucky,* Kingston*,* Litchfield*,* Newport*,* Oakey II*,* Ohio*,* Stanley*,* Typhi*, Salmonella,* Virchow*,* Wangata*,* Weltevreden*,* Westhampton*,* Westminster) *Salmonella spp, Shigella boydii 12, Shigella dysenteriae 12, Shigella flexneri 1b, Shigella sonnei, Vibrio fluvialis* and *Vibrio parahaemolyticus.*

### 2. Preferred antibiotics for the treatment of relevant pathogens

*K. pneumoniae*: Ceftriaxone, Ciprofloxacin, Levofloxacin, Imipenem, Colistin

*P. aeruginosa:* Piperacillin, Colistin, Tigecycline, Ciprofloxacin, Fosfomycin

*S. dysentery*: Ciprofloxacin, Pivmecillinam, Ceftriaxone

*S.* Typhimurium*:* Amoxicillin, Ampicillin, co-Trimoxazole

Enterotoxigenic *E. coli*: Ampicillin, Cephalosporin, Tetracycline, Sulphonamides

### 3. Antibiotic susceptibility tests

For the disc diffusion method, exponentially growing bacterial cells were seeded on Muller-Hinton agar (MHA, Difco, USA) plate (23” x 23” cm) using sterile cotton swabs and commercially available discs (BD, USA) containing defined amounts of interested antibiotics were placed on it. Plates were incubated overnight at 37°C in static incubator.

In the broth dilution method, cells were grown to log phage in Luria- Bertani(LB) broth or Brain Heart Infusion (BHI) media. Antibiotics were dissolved in water or organic solvents as per guidance of manufacturer (Sigma, USA). The zone of inhibition and minimum inhibitory concentration (MIC) of antibiotics for different bacterial strains were selected based on the previous reports (M45-2015, M45-2016, M100S-2016 and M100-S24-2014). MIC was used as the lowest concentration to completely inhibit visible growth of selected isolates after 24 h incubation at 37°C.

### 4. Next generation DNA sequencing

Genomic DNA from each of the XDR pathogens was used for rapid library preparation by adopting GS FLX+ sequencing chemistry (Roche, USA). Briefly, about 1 μg of genomic DNA was nebulized by nitrogen gas at a pressure of 30 psi for

2 minute for obtaining DNA fragments of length 900-1500 base pairs (bps). The nebulized DNA was purified by PCR Purification kit (Qiagen, USA), end polished with T4 DNA Polymerase, Polynucleotide Kinase and Taq DNA Polymerase (Roche, USA). Reactions were performed at 25ºC for 20 minutes and 72ºC for 20 minutes. Sequencing adaptor oligonucleotides was ligated to the polished DNA fragments using DNA Ligase (Roche, USA). Ligation reaction was performed at 25ºC for 10 minutes. The genomic DNA libraries thus obtained were then purified by commercially available Ampure XP cleanup kit (Beckman Coulter, USA). The quality of DNA libraries was analyzed using DNA High Sensitivity Chip in 2100 Bioanalyzer (Agilent, USA). The total amounts of dsDNA in the libraries were quantified using Picogreen dye in QubitFluorometer (Invitrogen, USA). About 140 x $10^7$ DNA library molecules per sample were clonally amplified by emulsion PCR in Mastercycler proS PCR systems (Eppendorf, Germany), purified using REMe integration (Roche, USA) on Biomek 3000 (Beckman Coulter, USA) and pyrosequenced in pico-titre plates in GS-FLX+ Genome Sequencers (Roche, USA). Sequence reads in FASTQ format were obtained from SFF files using sffinfo (GS-FLX) and a Linux script developed in-house. The sequence data generated per run were evaluated by FASTQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), using default parameters. In order to retain only the high quality reads in each metagenome, sequence reads having lengths less than 100 bps and at least one base with PHRAP quality scores less than 20, were removed from the metagenomic datasets.

**5. Statistical analysis**

For each association testing (resistance to a specific antibiotic in a specific pathogen or sampling center), 2X2 contingency tables were constructed using the four sets of values, namely the number of isolates having resistance to a given antibiotic in a group of interest (i.e. isolates from a specific species or sampled from a given center); number of isolates not having the corresponding resistance in that given group; number of isolates having resistance to the antibiotic but not belonging to the given group and; the number of isolates not having resistance to the antibiotic and not belonging to the concerned group. These values were then given as input to the 'chisq.test' functionality of R package v3.0.0 to obtain the statistical significance of the associations. Only those associations having a p-value of less than 0.05 were then reported.

Furthermore, the correlation patterns across resistances were obtained by first calculating the detection percentages of the resistances to various antibiotics in the isolates belonging to the different pathogenic species across the different years and then computing the Pearson correlations of these detection percentages across the different years (using core functionality of R package v3.0.0).

**6. Engineering *hapR* positive whole genome sequenced *V. cholerae* cell**

First, N16961Δ*hapR*::*ble* strain SB6 was engineered by using recombinant vector pAP5, a derivative of suicide plasmid pKAS32 carrying *sh ble* gene flanked by the upstream and downstream region of *hapR* allele. Then, Δ*hapR*::*ble* allele was replaced from the chromosome of SB6 using recombinant vector pAP6, derivative of pKAS32 carrying functional *hapR* allele from *V. cholerae* strains C6709. The gene replacement method was adopted as described previously (Das et al, 2009). Genotype and phenotype of *hapR* positive strains SB8 were confirmed by PCR amplification and HA protease activity measurement (Supl. Fig. S2) as reported previously (Syngkon et al. 2010).

**References:**


1. Das B, Pal RR, Bag S, Bhadra RK. Stringent response in *Vibrio cholerae*: genetic analysis of spoT gene function and identification of a novel (p)ppGpp synthetase gene. *Mol Microbiol* 2009; **72**(2): 380-98.
2. Syngkon A, Elluri S, Koley H, et al. Studies on a novel serine protease of a DeltahapADeltaprtV Vibrio cholerae O1 strain and its role in hemorrhagic response in the rabbit ileal loop model. *PloS one* 2010; **5**(9).

**Legends to the supplementary information:**

**Supl. Information S1:** Details of the materials and methods including isolation and cultivation of bacterial species, antibiotic susceptibility tests, genomic engineering, next generation DNA sequencing, genome assembly, gene annotations, brief description about the relevant bacterial strains and mobile genetic elements, plasmid curing experiment and statistical analysis used in this study are provided in the supplementary information S1.

**Supl. Table S1:** Name of the bacterial isolates, isolation ID, date of isolation and susceptibility to different antibiotics are mentioned in the supplementary Table S1.

**Supl. Table S2:** Resistance profile of 27 XDR isolates against 22 antibiotics representing nine distinct drug classes are mentioned in the supplementary Table S2. Whole genome sequenced XDR isolates are highlighted in boldface.

**Supl. Table S3:** Genomic information of six whole genome sequenced XDR pathogens.

**Supl. Table S4:** List of antibiotics (for different species) for which zone of inhibition and minimum inhibitory concentration interpretive standards are available or not available in CLSI.

**Supl. Table S5:** Details of the plasmids, antibiotic concentration, functional evaluation of resistance genes, primer sequences, β-lactamase enzyme classes are provided in the supplementary Table S5.

**Supplementary Table S 5A:** Several β-lactamase enzymes detected in the sequenced genome of XDR isolates

**(i).** β-lactamase enzymes present in the genome of XDR *Providencia stuartii* MV493

| Name | Size (aa) | Class | Closest member |
|---|---|---|---|
| Beta-lactamase (EC 3.5.2.6) | 380 | C | *Providencia rettgeri* (Id, 92%; Sim 96%) |
| Beta-lactamase (EC 3.5.2.6) | 381 | C | *Salmonella enterica* (Id, 99%; Sim 100%) |
| Beta-lactamase (EC 3.5.2.6) | 295 | A | *Escherichia coli* (Id, 100%, Sim, 100%) |
| Beta-lactamase | 270 | B | *Acinetobacter baumannii* (Id, 100%, Sim, 100%) |

**(ii).** β-lactamase enzymes present in the genome of XDR *Klebsiella pneumoniae* MV36808

| Name | Size (aa) | Class | Closest member |
|---|---|---|---|
| β-lactamase (EC 3.5.2.6) | 286 | A | *Enterobacteriaceae* (Id, 100%; Sim, 100%) |
| β-lactamase | 428 | C | *Enterobacteriaceae* (Id, 99%; Sim, 99%) |
| β-lactamase | 286 | A | *Escherichia coli* (Id, 100%; Sim, 100%) |
| β-lactamase | 270 | B | *Acinetobacter baumannii* (Id, 100%; Sim, 100%) |
| β-lactamase | 338 | C | *Enterobacter hormaechei* (Id, 100%; Sim, 100%) |
| β-lactamase | 291 | A | *Pantoea agglomerans* (Id, 100%; Sim, 100%) |
| β-lactamase | 295 | A | *Escherichia coli* (Id, 100%; Sim, 100%) |
| β-lactamase | 291 | D | *Escherichia coli* (Id, 99%; Sim, 99%) |

**(iii).** β-lactamase enzymes present in the genome of XDR *Pseudomonas aeruginosa* 36846

| Name | Size (aa) | Class | Closest member |
|---|---|---|---|
| β-lactamase | 610 | C | *Pseudomonas sp.* (Id 98%, Si 98%) |
| β-lactamase | 262 | D | *Pseudomonas aeruginosa* (Id 100%, Si 100%) |
| β-lactamase | 371 | C | *Pseudomonas otitidis* (Id 99%, Si 99%) |

| β-lactamase | 397 | C | *Pseudomonas aeruginosa* (Id 99%, Si 100%) |
|---|---|---|---|
| β-lactamase | 381 | C | *Pseudomonas aeruginosa* (Id 99%, Si 99%) |
| β-lactamase | 391 | C | *Pseudomonas aeruginosa* (Id 99%, Si 100%) |

**(iv).** β-lactamase enzymes present in the genome of XDR *Escherichia coli* MV292587

| Name | Size (aa) | Class | Closest member |
|---|---|---|---|
| β-lactamase | 377 | C | *E. coli* (Id 100%, Si 100%) |
| β-lactamase | 433 | C | *E. coli* (Id 100%, Si 100%) |
| β-lactamase | 286 | A | *Klebsiella pneumoniae* (Id 100%, Si 100%) |
| β-lactamase | 291 | A | *Pantoea agglomerans* (Id 99%, Si 100%) |
| β-lactamase | 291 | D | *Serratia marcescens* (Id 99%, Si 99%) |

**(v).** β-lactamase enzymes present in the genome of XDR *Shigella flexneri* MV07210

| Name | Size (aa) | Class | Closest member |
|---|---|---|---|
| β-lactamase | 376 | C | *Shigella flexneri* (Id 99%, Si99%) |
| β-lactamase | 291 | D | *Serratia marcescens* (Id 99%, Si 99%) |
| β-lactamase | 249 | A | *E. coli* (Id 99%, Sim 99%) |

**(vi).** β-lactamase enzymes present in the genome of XDR *Salmonella* Typhimurium MV32691

| Name | Size (aa) | Class | Closest member |
|---|---|---|---|
| β-lactamase | 293 | B | *Salmonella enterica* (Id 100%, Si 100%) |
| β-lactamase | 432 | C | *Salmonella enterica* (Id 100%, Si 100%) |

**Supplementary Table S 5B:** Relevant plasmids used in this study

| Plasmids | Genotype/Phenotype | References |
|---|---|---|

| pBD62 | pSW23T *sh ble ori*R6K, *mob*RP4, $attP^{ctx}$, $Zeo^R$ | Das et al. 2014 |
|---|---|---|
| pBAD24 | pBR322 *ori; araC, bla;* $Amp^R$ | Das et al. 2014 |
| pKAS32 | *Ori*R6K, *mob*RP4, *rpsL, bla*, conjugative vector; $Amp^R$ | Das et al. 2009 |
| pAP3 | pUC18:: *hapR* region, $Amp^R$ | This study |
| pAP4 | pUC18:: Up-Dw Δ*hapR:sh ble* | This study |
| pAP5 | pKAS32::Up-Dw Δ*hapR:sh ble* | This study |
| pAP6 | pKAS32:: *hapR* region, $Amp^R$ | This study |

**Supplementary Table S 5C:** Evaluation of function of predicted ORFs. All the ORFs with putative antimicrobial resistance functions were cloned under the control of $P_{BAD}$ promoter in pBD62 and/or pBAD24 vector and resistance functions were examined in the complemented host by broth dilution and/or disk diffusion assays.

*Amp- Ampicillin; Azt- Aztreonam; Car- Carbenicillin; Far- Faropenem; Fos-Fosfomycin; Gen- Gentamycin: Imp-Imipenem; Kan-Kanamycin; Ksg- Kasugamycin; Neo- Neomycin; Pen-Penicillin; PB -Polymyxin B; Spt-Spectinomycin; Str-streptomycin Tet-Tetracycline; Zeo- Zeocin
RE- Restriction endonuclease

| Name | Vector backbone | Insert | Host | Pri mers used | RE used | Function |
|---|---|---|---|---|---|---|
| ***K. pneumoneae* (MV36808)** | | | | | | |
| pBIP27 | pBD62 | $bla_{NDM1}$ | FCV14 | 681, 682 | EcoRI, XbaI | Resistant: Amp25, Imp5.0<br>Sensitive: Azt1.6 |
| ***Providencia stuartii* (MV493)** | | | | | | |
| pPD1 | pBAD24 | $bla_{NDM}$ | FCV14 | 418, 419 | EcoRI, PstI | Resistant: Imp 10<br>Sensitive: Azt1.6 |
| pPD2 | pFX497 | $bla_{NDM}$ | FCV14 | 443, 444 | HindIII. SacI | Resistant: Imp 10<br>Sensitive: Azt1.6 |
| pBIP10 | pBIP11 | Aph3 | DH5α | 458, 459 | EcoRI, PstI | Resistant: Kan50, Gen1.5<br>Sensitive: Gen2.5/5 str50 |
| pBIP14 | pBIP11 | *ksgA* | FCV14 | 452, 453 | EcoRI, NsiI | Sensitive: Ksg50 |
| pBIP15 | pBIP11 | *aph3'* | DH5α | 454, 455 | EcoRI, PstI | Sensitive: Kan50, Gen5, str50 |
| pBIP16 | pBIP11 | *bl2be_shv2* | FCV14 | 456, 457 | EcoRI, NsiI | Resistant: Amp100 |
| pBIP17 | pBIP11 | $bla_{NDM}$ | FCV14 | Sub cloned from pPD2 | HindIII, SacI | Resistant: Amp100, Imp10<br>Sensitive: Azt1.6 |
| pBIP18 | pBIP11 | *fosA* | FCV14 | 482, 483 | HindIII, PstI | Sensitive: Fos4 |
| pBIP19 | pBIP11 | *emrE* | FCV14 | 484, 485 | EcoRI, PstI | Sensitive: Kan50, Gen5, str50 |
| pBIP20 | pBIP11 | metallo-blb gene | FCV14 | 486, 487 | EcoRI, PstI | Sensitive: Imp1 |
| pBIP21 | pBIP11 | *tet34* | FCV14 | 490, 491 | EcoRI, PstI | Sensitive: Tet 1.5 |

**Supplementary Table S 5D:** List of antibiotics, abbreviation and minimal inhibitory concentration (MIC) used in this study

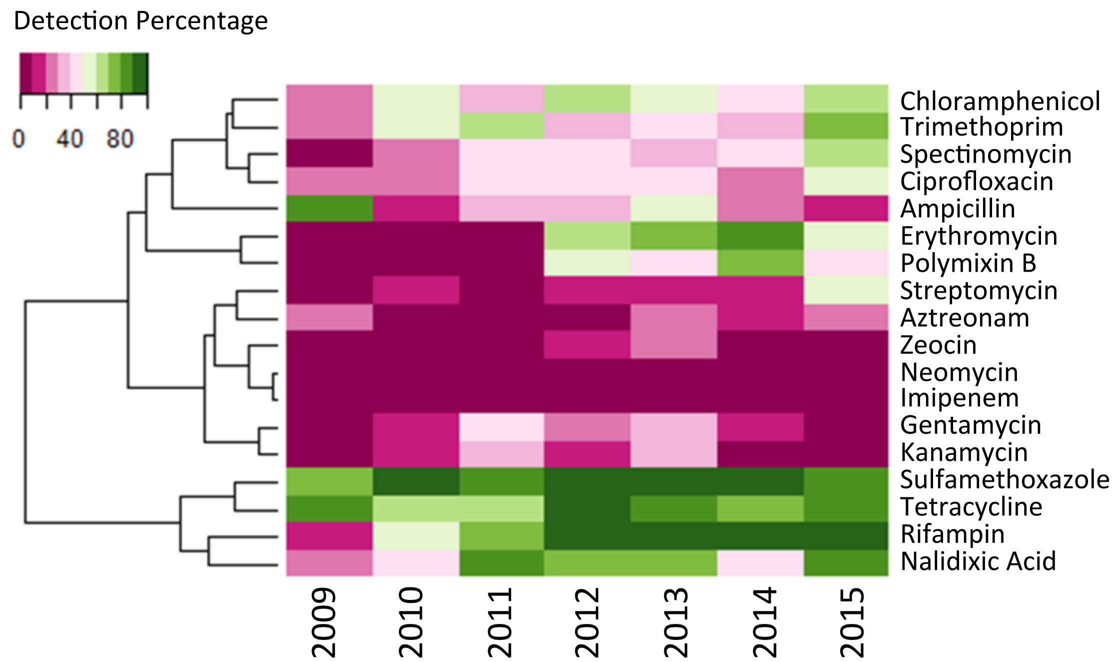
| **Antibiotic** | **Chemical class** | **Antibiotic abbreviation** | **MIC** | |
|---|---|---|---|---|
| | | | **Solution (µg/ml)** | **Disc** |
| Ampicillin | β-lactam | Amp | 10 | 10 |
| Penicillin | β-lactam | Pen | - | 10 |
| Carbenicillin | β-lactam | Car | - | 100 |
| Imipenem | β-lactam | Imp | 10 | 10 |
| Faropenem | β-lactam | Far | 4/10/130 | - |
| Aztreonam | β-lactam | Azt | 1.6 | - |
| Polymixin B | polypeptide | PB | 50 | 300 |
| Kanamycin | Aminoglycoside | Kan | 30/40 | 30 |
| Spectinomycin | Aminoglycoside | Spt | 40 | 15 |
| Streptomycin | Aminoglycoside | Str | 100 | 10 |
| Neomycin | Aminoglycoside | Neo | 40 | 40 |
| Gentamicin | Aminoglycoside | Gen | 10 | - |
| Chloramphenicol | Chloramphenicol | Chl | 3/30 | 30 |
| Tetracycline | Tetracycline | Tet | 1.5/5 | 5 |
| Doxycycline | Tetracycline | Dox | - | 30 |
| Erythromycin | Macrolide | Ery | 100 | 100 |
| Rifampin | Ansamycins | Rif | 2/5 | 5 |
| Ciprofloxacin | Quinolones | Cip | 4/30 | 5 |
| Nalidixic acid | Quinolones | Nal | 4/30 | 30 |
| Zeocin | Glycopeptide | Zeo | 25 | - |
| SXT | Sulfonamide-Pyrimidine | SXT | - | Sulfamethoxazole 23.75 Trimethoprim 1.25 |
| Trimethoprim | Pyrimidine | Tmp | 30 | - |
| Sulfamethoxazole | Sulfonamide | Sss | 160 | - |

**Supplementary Table S 5E:** Primers used in this study.

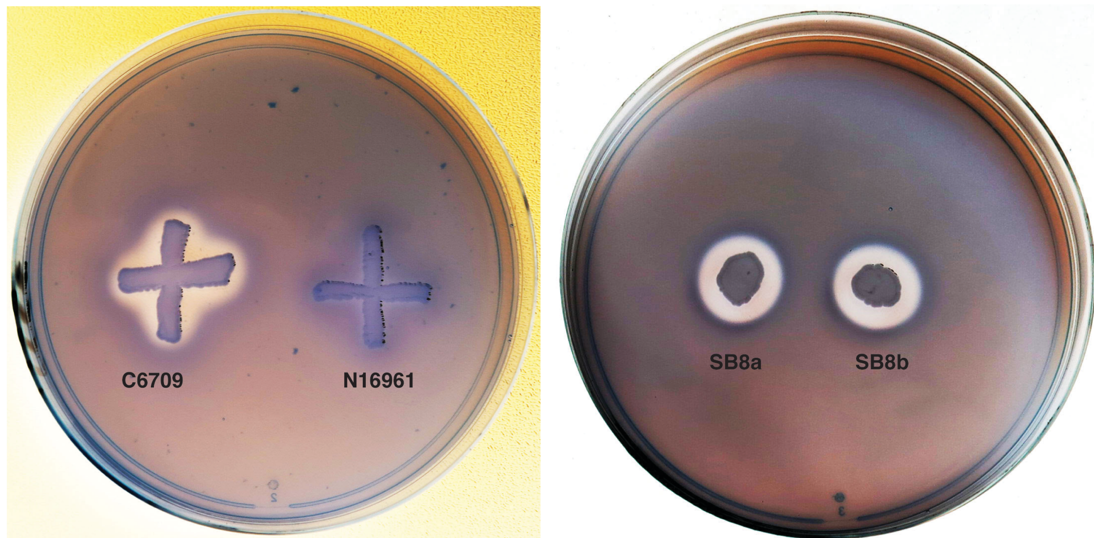
| **Primer Name** | **Primer sequence** | **Target gene** |
|---|---|---|
| 418 | CCGAATTCACCATGGAATTGCCCAATATTATG | *bla*$_{NDM}$ *P. stuartii* |
| 419 | GGCTGCAGTCAGCGCAGCTTGTCGGCCATG | *bla*$_{NDM}$ *P. stuartii* |
| 428 | GCGAGCTCTGGTGGTTCATAGCCATGAG | *hapR* gene region of C6709 |
| 429 | CCTCTAGAATCATCGCCTACTGTGTC | *hapR* gene region of C6709 |
| 443 | GGAAGCTTTGACCTATGACCGTGGACGTGAG | *bla*$_{NDM}$ gene of *P. stuartii* |
| 444 | GGGAGCTCTCAGCGCAGCTTGTCGGCCATG | *bla*$_{NDM}$ gene of *P. stuartii* |
| 448 | CCGAATTCGTCACATTCAATTCAATTCC | *bla* gene of *P. stuartii* |
| 449 | GGCTGCAGGTATTACTGGTTCGTTATCTG | *bla* gene of *P. stuartii* |
| 450 | GGGGATCCGATTACGGTTGTATGCTACG | Polymyxin resistance gene of *P. stuartii* |
| 451 | CCCTGCAGCCTTGGTAGGTATCCACATC | Polymyxin resistance gene of *P. stuartii* |
| 452 | CCGAATTCCCACGACACATCTGCCACTC | kasugamycin resistance gene of *P. stuartii* |

| 453 | CCATGCATCGCGCAATATCGGGTTGAG | kasugamycin resistance gene of *P. stuartii* |
|---|---|---|
| 454 | CCGAATTCGCTTATAGCAGTGTCACAG | *aph* gene of *P. stuartii* |
| 455 | CCCTGCAGGGTGGTTTATGTCGCACTTC | *aph* gene of *P. stuartii* |
| 456 | CCGAATTCGGAAATTGCTCATCAGCTCAG | *bla* gene of *P. stuartii* |
| 457 | GGATGCAGGAGTAAACTTGGTCTGACAG | *bla* gene of *P. stuartii* |
| 458 | CCGAATTCCCTCTGATGTTACATTGCAC | *aph* gene of *P. stuartii* |
| 459 | CCCTGCAGGCTCTGCCAGTGTTACAACC | *aph* gene of *P. stuartii* |
| 482 | GGCTGCAGGGAGGTAGGTGGTTACGATACTG | *fosA* gene of *P. stuartii* |
| 483 | CCAAGCTTTATACAACTCTGCGTAAAGC | *fosA* gene of *P. stuartii* |
| 484 | GGGAATTCCAGATGCCACCGACTTGGCAATG | *emre* gene of *P. stuartii* |
| 485 | CCCTGCAGCTGCCTGAATATTCTGCACC | *emre* gene of *P. stuartii* |
| 486 | GGCTGCAGCGTTGCGAAGAAATGACAAG | $bla_{NDM1}$ gene of *P. stuartii* |
| 487 | CCGAATTCCTCGTGAATTGCAGTCGTCC | $bla_{NDM1}$ gene of *P. stuartii* |
| 490 | CCGAATTCGACCGAGCAACTCAAATTAC | *tet34* gene of *P. stuartii* |
| 491 | GGCTGCAGGTCTATCGGAGTTGAATTTGG | *tet34* gene of *P. stuartii* |
| 492 | GGGAATTCGCAAGGTAGCCAACTAAAGG | *rosa* gene of *P. stuartii* |
| 493 | CCCTGCAGGAGATCGTCATCAAATAATTAACC | *rosa* gene of *P. stuartii* |
| 681 | CCGAATTCATGAAATTATCTGCCCTTGC | $bla_{NDM}$ gene of *K. pneumoneae* |
| 682 | CCTCTAGATCACCACTTCATCTCACC | $bla_{NDM}$ gene of *K. pneumoneae* |

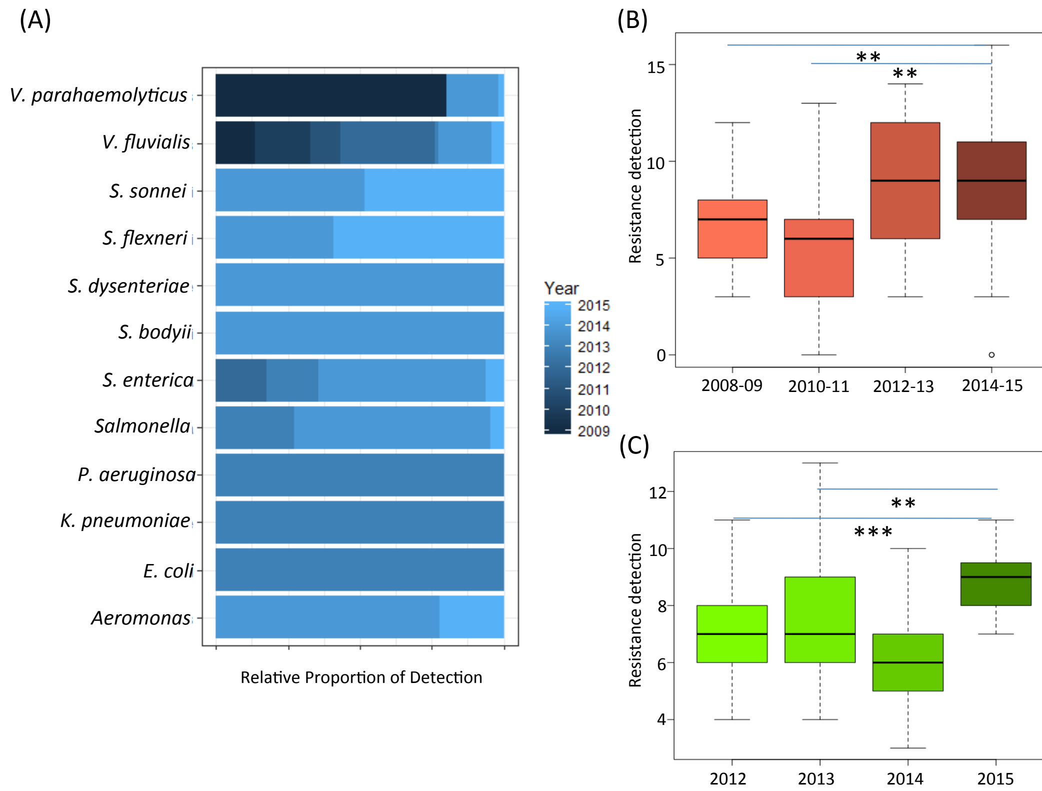
## Supplementary Figures




**Supl. Figure S1:** Detection percentage of resistance to various antibiotics across the years 2009-2015.




**Supl. Figure S2:** Protease assay of *hapR* positive *V. cholerae* strains SB8 derived from whole genome sequenced strain N16961. Zone of clearance of skim milk protein in the minimal medium indicate expression and secretion of extracellular protease modulated by transcriptional factor HapR.




**Supl. Figure S3:** (A): Detection pattern of various pathogens across the years from 2009-2015 (B) Resistance detection (i.e. the number of antibiotics against which resistance was detected) of the isolates of *V. fluvialis* across the years. (C) The number of antibiotics against which resistance was detected for the isolates of *S. enterica* obtained from 2012-15. Significant differences between the current and earlier years are highlighted with *s. ** indicates P-value < 0.01 and *** indicates P-value < 0.001.