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Original Article

# IDENTIFICATION OF GENES/ ENZYMES RESPONSIBLE FOR ANTIBIOTIC RESISTANCE IN *VIBRIO ALGINOLYTICUS* STRAIN PTS13

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**ABSTRACT**

*Vibrio alginolyticus* is a halophilic Gram-negative pathogenic bacterium. It is considered as one of the most frequent species living freely in water and sediment and can survive in sea water even in famine conditions while maintaining their virulence. Gram-negative bacteria are highly efficient in up-regulating or acquiring genes that code for mechanisms of antibiotic resistance, especially in the presence of antibiotic selection pressure. Furthermore, they have available to them a plethora of resistance mechanisms, often using multiple mechanisms against the same antibiotic or using a single mechanism to affect multiple antibiotics. This study began with the observation of multi-resistant *Vibrio alginolyticus* strain recovered from the coastal sediment of Tuticorin beach. The aim of this study was to determine the prevalence of some important resistance genes encoding *bla* TEM, *bla* CTX-M, *gyr*A and *bla* VIM in the test isolate i. e. *Vibrio alginolyticus* strain PTS13.

**Keywords:** *Vibrio alginolyticus*, Antibiotic, Resistance, *Bla* TEM, *Bla* CTX-M, *gyr*A, *Bla* VIM.

## INTRODUCTION

### Antibiotic

Since the discovery of inhibitory properties of antibiotics they have been widely used for treating bacterial infections [1]. These drugs are natural or synthetic or semi synthetic substances capable of killing bacteria or retarding their growth [2]. These are mainly used for treating human infections but their use is not restricted to medical purposes as they are also extensively applied in animal husbandry for veterinary objectives and growth promoters as well as in agriculture and aquaculture [3]. The main target sites of antimicrobial agents are; (i) Cell wall synthesis, (ii) Protein synthesis, (iii) Nucleic acid synthesis, (iv) Metabolic pathways, and (v) Cell membrane functions [4]. However, the effectiveness of treatments based on antibiotic therapy has been reduced over time, as a result of the spread of antibiotic resistant bacteria [2].

### Details of the different antibiotics used in the study

### β-lactam antimicrobial agents

β-lactam antimicrobial agents are a large family of bactericidal agents consisting of penicillins, cephalosporins, cephamycins, carbapenems, and monobactams. Ampicillin is a semisynthetic β-lactam antibiotic that has been commercially used on a global scale since the 1960s. The structure of the β-lactams varies around the β-lactam ring, a consistent structure of the class.

(a) Penicillins have a five-membered ring attached to the β-lactam ring;

(b) Cephalosporins have a six-membered ring in addition to differing side chains attached to the rings [4];

(c) Carbapenems are the last developed group of β-lactams; they are used as the last choice against bacterial infections when treating serious infections caused by multi-drug resistant pathogens [5].

### Mechanism of action

β-lactams have the ability to bind to the membrane proteins in the bacterial cell wall like carboxypeptidases, transglycosylases and transpetidases, which function in the cross-linking of two glycan-linked peptide chains. Hence the name "penicillin binding proteins" or PBPs. Without this cross-linking, the cell wall loses its stability and strength and will therefore result in cell lysis. When penicillin binds to the PBPs they inhibit the cross-linking reaction leading to an accumulation of precursor cell wall units and to an activation of the autolytic system, resulting in cell lysis [4, 6, 7].

### Vancomycin

It was introduced as an antimicrobial agent in the late 1950s. It belongs to the glycopeptide family of antibiotics which inhibit the peptidoglycal synthesis.

### Mechanism of action

It blocks the peptidoglycan crosslinking by binding to the D-Ala-D-Ala termini of the immature peptidoglycan. Resistant bacteria synthesize a modified immature peptidoglycan with D-Ala-D-lactate termini, which is poorly bound by vancomycin, allowing completion of cell wall synthesis [8]. There were first reports of Vancomycin resistant Enterococcus (VRE) in 1988 [9, 10].

Quinolones are synthetic broad-spectrum antibiotics and are widely used in clinical practice and aquaculture.

### Mechanism of action

They inhibit DNA gyrase of Gram-negative bacteria or topoisomerase IV enzyme of Gram-positive bacteria that are required for bacterial DNA synthesis [11]. They have a wide spectrum of activity and have been extensively used and misused in both human and veterinary medicine leading to its resistance. The fluoroquinolone ciprofloxacin has been pointed out as the most consumed antimicrobial agent worldwide [12].

### Antimicrobial resistance

The increase of bacterial resistance to antimicrobial agents in our society is a problem caused by many factors. From the bacterial point of view, resistance in many cases means survival and is highly valued. Bacterial strains have evolved numerous strategies to avoid antimicrobial agents like producing enzymes that inactivate or modify antimicrobial agents, have an altered binding site for the antimicrobial agent, or restrict access to the target by reduced permeability or efflux mechanisms [4].

A key factor in the development of antibiotic resistance is the ability of microorganisms to adapt quickly to new environmental conditions. As bacteria are single-celled organisms with small numbers of genes, even a single random genetic mutation can greatly affect their ability to cause disease. And because most microbes reproduce by dividing every few hours, a mutation that helps a microbe surviving to an antibiotic exposure will quickly become dominant throughout the microbial population. Also microbes often acquire resistance genes from each other through horizontal gene transfer mechanism which might enable them to be

a multiple antibiotic resistant strain. The specificity of the interactions between antibiotics and various protein sequences within a bacterium resulting in significantly high ratio of mutations in its genome as well as a particular mutation in a certain target sequence will result in antibiotic resistance [13].

**Mechanisms of resistance**

Resistance mechanisms can either be an intrinsic property of a bacterial species or an acquired trait. Acquired resistance occurs as a result of chromosomal mutations or by the acquisition of genetic elements. Intrinsic resistance covers a whole bacterial species and provides resistance without the addition of genetic elements or mutations.

**β-lactam resistance mechanisms**

Resistance to β-lactams is generally the result of the following three mechanisms [4, 5].

1. Resistance by alteration in target site.

2. Resistance by alteration in access to the target site.

3. Resistance by production of β-lactamases.

**β-lactamases**

These enzymes able to hydrolyze penicillin were identified in *E. coli* as early as in 1940, before penicillin was taken into clinical use, proving a natural origin [5]. The β-lactamases execute their effect in hindering the work of β-lactams by hydrolyzing the β-lactam ring structure [4, 5].

The enzymes can be encoded on both plasmids and chromosomes [4, 5, 14, 15], and are highly selected for by the extensive use of β-lactams in our society. The plasmid-encoded β-lactamases are related to the chromosomally-encoded enzymes [16]. β-lactamases can be transferred within different bacteria [14, 15]. The β-lactamases are located differently in Gram-positive and Gram-negative bacteria. The enzymes are mostly extracellular in Gram-positive bacteria, while periplasmic in Gram-negative species. There has been identified over 890 β-lactamases targeting various β-lactam antibiotics [4, 5].

**Classification of β-lactamases**

It can be done either according to the functional properties (Bush-Jacoby -Medeiros scheme) or by the molecular class (Ambler classification scheme) [17, 18].

The functional classification is based on the substrate profile of the enzyme and its inhibitory profile. It divides the β-lactamases into three groups. Group 1 is cephalosporinases; Group 2 consists of broad-spectrum, inhibitor-resistant, and extended-spectrum beta-lactamases and serine carbapenemases; Group 3 holds the metallo-β-lactamases.

The molecular classification is based on the sequence similarities and catalytic mechanisms. Class A, C, and D all have a serine at their active site and Class B has a zinc based active site.

**(i) β-lactamase TEM**

The most common resistance mechanisms towards ampicillin is the production of β-lactamases encoded by *bla* TEM alleles [19]. TEM β-lactamase, was reported in gram-negative bacteria containing multiple drug resistant R-plasmids that date from 1962 [20]. This enzyme became widespread throughout the world, making penicillins with gram-negative activity, such as ampicillin, almost useless [21]. A large number of TEM variants have been described (>130) where single amino acid substitutions occur at a limited number of positions [22]. The enzyme confers resistance to penicillins like ampicillin, ticarcillin, and piperacillin, and narrow-spectrum cephalosporins, like cephalothin or cefazolin [19].

The first Class A beta-lactamase from a Gram-negative bacterium was the TEM beta-lactamase of *Eschericia coli*, named for the patient from which it was isolated [20, 23]. The Class A enzymes TEM & SHV (sulfhydryl variant of TEM) can be found in the chromosomal DNA of their host bacteria, or on mobile genetic elements called plasmids, which bacteria can share freely with one another [24, 27].

**(ii) Extended-spectrum β-lactamases (ESBLs)**

The extensive use of the third generation cephalosporins like cefotaxime, ceftriaxone and ceftazidime has led to the evolution of newer β-lactamases such as the Extended Spectrum β- Lactamases (ESBLs). ESBLs are plasmid mediated enzymes that hydrolyze the oxyimino β-lactams and monobactams (aztreonam) but have no effect on the cephamycins (cefoxitin, cefotitan) and the carbapenems (Imipenem). Being plasmid mediated, they can be easily transferred from one bacterium to another [28].

ESBLs were first described in Germany in 1985 [24]. They are defined as enzymes with the ability to hydrolyse extended-spectrum cephalosporins and are inhibited by clavulanate. The original ESBLs were point-mutation derivatives of the restricted-spectrum TEM and SHV enzymes commonly found in ampicillin resistant *E. coli* and Klebsiella spp [22].

**CTX-M β-lactamase**

Especially troublesome among the ESBL enzymes are those called CTX-M [14]. This ESBL hydrolyze β-lactam antibiotics containing the oxymino side-chain [29]. This enzyme confer a high-level resistance to cefotaxime [14, 30, 31], cefriaxone [14, 31] and aztreonam [32] but is weakly active against ceftazidime [14, 30, 31].

Although most ESBLs are mutants of TEM and SHV enzymes, the CTX-M type β-lactamases originated from β-lactamases found in the chromosome of the environmental species of the genus *Kluyvera* [14, 31] and have transferred to R plasmids and consequently the enzyme rapidly became widespread among R-plasmid-containing pathogenic bacteria [33].

Particular attention should be paid to the worldwide increasing prevalence of the CTX-M types among the different ESBLs as these enzymes are prevalent not only in nosocomial environment, but also in the community setting [34-40].

**(iii) Metallo-betalactamases (MBLs)**

Class B carbapenemases termed as metallo-β-lactamases (MBLs) present one or two zinc ions in their active site, which bond to water molecules that intervene in the hydrolytic inactivation of the beta-lactam ring [41-44]. These are able to hydrolyse even the carbapenems [45].

Genes encoding for MBLs were shown to be carried on large transferable plasmids or were associated with transposons, allowing horizontal transfer of these MBL genes among different bacterial genera and species [46]. These enzymes are active against carbapenems but they do not hydrolyse monobactams. They are inhibited by metallic ions' chellant agents such as EDTA; they are not inhibited by clavulanic acid, sublactam or tazobactam [41- 44].

To date five types of acquired MBL genes (IMP, VIM, SPM, GIM, and SIM) have been identified based on their divergent protein molecular structures [47]. IMP and VIM variants have been reported worldwide while members of SPM, GIM, and SIM are restricted to certain geographical regions [48].

The VIM-type metallo-betalactamases exhibit a wide spectrum of hydrolytic activity against beta-lactam antibiotics, including carbapenems and their respective *bla* genes are located in the variable region of class 1 integrons [49, 50].

**Antibiotic resistance by mutations in the target**

**Fluoroquinolone resistance**

Resistance to quinolones has been reported in a variety of important bacterial pathogens [51, 52]. A line of evidence has indicated that bacteria resist the action of the quinolones mainly through two types of mechanisms:

(1) Modification or mutation of DNA gyrase or topoisomerase decreases their binding affinity to quinolones [53, 54].

(2) Activation of efflux pumps and regulation of outer membrane permeability result in decrease of intracellular quinolone concentration [52, 55].

Mutations in the target area where the quinolones attach confer resistance [4]. These mutations are often seen within conserved regions of two genes, *gyr*A and *par*C. The genes encode, respectively, the A subunit of DNA gyrase and the homologous A subunit of to poisomerase IV [56, 57]. The conserved regions within the genes were named "Quinolone Resistance Determining Regions", shortened to QRDR [57]. Likewise, characterization of the QRDRs of gram-negative and gram-positive bacteria may be accomplished by amplification of the *gyr* A, *gyr* B, *par* C,and *par* E genes by PCR, followed by sequencing of the amplicons to detect point mutations [58, 59].

**About the species**

*Vibrio alginolyticus* is a halophilic Gram-negative pathogenic bacteria. It is considered as one of the most frequent species living freely in water and sediment [60] and can survive in sea water even in famine conditions while maintaining their virulence [61]. Infections caused by gram negative bacteria have features that are of terrible concern. These organisms are highly efficient in up-regulating or acquiring genes that code for mechanisms of antibiotic resistance, especially in the presence of antibiotic selection pressure. Furthermore, they have available to them a plethora of resistance mechanisms, often using multiple mechanisms against the same antibiotic or using a single mechanism to affect multiple antibiotics [37].

This study began with the observation of multi-resistant *Vibrio alginolyticus* strain recovered from the coastal sediment of Tuticorin beach which is an alarming finding. The aim of this study was to determine the prevalence of some important resistance genes encoding *bla* TEM, *bla* CTX-M, *gyr* A and *bla* VIM in the test isolate i. e. *Vibrio alginolyticus* strain PTS13.

## METHOD AND MATERIALS

**Description of the Study Area**

The particular sample was collected from New harbor beach, Tuticorin (Lat. 8°44'N; Long. 78°10'E).

**Sampling**

Sediment samples from the coastal region were collected in pre-sterilized tubes using PVC corer.

**Isolation of bacteria**

Serially diluted samples were spread plated on Zobell 2216 marine agar [62] containing ampicillin (100µg/ml) for primary screening. The occurrence of antibiotic resistant bacteria was determined by the disc diffusion method using Mueller-Hinton agar, according to the Bauer-Kirby method [63] as described by the National Committee for Clinical Laboratory Standards. The clinical antibiotics belonging to different classes of antibiotics (Himedia) used in this study are listed in table 1.

**Table 1: List of clinical antibiotics and their concentration**

| S. No. | Antibiotic | Concentration | S. No. | Antibiotic | Concentration |
|---|---|---|---|---|---|
| 1. | Amikacin | 10/30 mcg | 9. | Imipenem | 10 mcg |
| 2. | Amoxyclav | 30 mcg | 10. | Imipenem/Cilastin | 10/10 mcg |
| 3. | Azithromycin | 15 mcg | 11. | Levofloxacin | 5 mcg |
| 4. | Cefotaxime | 10/30 mcg | 12. | Meropenem | 10 mcg |
| 5. | Ciprofloxacin | 10 mcg | 13. | Penicillin | 10 units |
| 6. | Co-trimoxazole | 25 mcg | 14. | Polymyxin-B | 300 units |
| 7. | Doxycycline | 10/30 mcg | 15. | Rifampicin | 5/15 µg |
| 8. | Gentamicin | 30 µg | 16. | Vancomycin | 30 mcg |

Approximately 140 bacterial strains were isolated after from seven different coastal regions of Tamilnadu, among which 12% strains were found to be multi drug resistant. Among them, this strain PTS13 later identified as *Vibrio alginolyticus,* isolated from the sediment of Tuticorin coastal region was selected for this study as it showed resistance against maximum number of antibiotics i. e. ten different antibiotics belonging to different classes.

**Molecular Identification of strain**

Genomic DNA isolation: Genomic DNA of overnight grown bacterial broth cultures was isolated using the Phenol-chloroform method as defined by Sambrook & Ressel [64].

16S rRNA amplification: It was done as described by Saiki *et al.* [65] to confirm the bacterial strain identification [66] by using the 16S rRNA universal primers namely 8F (5'-AGA GTT TGA TCC TGG CTC AG-3') & 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') using Mastercycler gradient (Eppendorf, Germany). Components for PCR master mix like PCR buffer; deoxynucleotides (dATP, dCTP, dGTP, & dTTP); *Taq* polymerase and oligoprimers (8F & 1492R) were purchased from Sigma-Aldrich (USA). Amplification of 16S rRNA genes by the polymerase chain reaction provides access to these genes for sequence analysis.

16S rRNA sequence analysis: 16S rRNA amplified products were sent to Amnion Biosciences Pvt. Ltd. (Karnataka) for their sequence analysis in order to identify the bacterial strain.

**Biochemical characterization of strain**

The biochemical tests applied for the species confirmation were:

(a) Gram staining

(b) Growth on thiosulphate-citrate-bile salt-sucrose (TCBS) agar

(c) Glucose fermentation

(d) Sucrose fermentation

(e) Lactose fermentation

(f) Hydrogen sulphide production

(g) Bioluminescence

(h) Growth in NaCl

(i) Motility agar test/ Stabbing technique method

(j) Triple sugar iron test

**Quantification of genomic DNA**

The quantification of genomic DNA in the test strain *Vibrio alginolyticus* strain PTS13 was done with the use of Qubit Fluorometer.

**Amplification of genes encoding for resistance**

PCR amplification was done for detection of resistance genes like *bla* CTX-M, *gyr*A, *bla* TEM and *bla* VIM. The PCR was performed in a Mastercycler gradient (Eppendorf, Germany) after which amplified DNA products were resolved by electrophoresis in 1% agarose (w/v) gels in TAE buffer (pH 7.0) containing 0.5 mg of ethidium bromide per liter and photographed with Alpha imager EC (Alpha Innotech, USA). Either the 1-kb DNA ladder or the 100-bp DNA ladder (Sigma Aldrich) was used to assess PCR product size. Components for PCR mastermix were purchased from Sigma-Aldrich (USA).

**Amplification of *bla* CTX-M gene**

The oligonucleotide primers used for PCR assay were described by Yang *et al.* [67].

*bla*CTX-M F 5'-CGCTTTATGCGCAGACGA-3'

*bla*CTX-M R 5'-GATTCTCGCCGCTGAAGC-3'

The PCR was performed in 50 µl volumes containing 50 ng of DNA, 25 pM each of primer, 100 µM dNTPs, 2.5 U of *Taq* Pol and PCR buffer (containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2 and 0.001% gelatin). The PCR conditions were as follows: initial denaturation at 94°C for 10 min; denaturation of 94°C for 1 min for 35 cycles; annealing at 55°C for 1 min; extension at 72°C for 3 min and final extension at 72°C for 10 min.

**Amplification of *gyrA* gene**

The oligonucleotide primers used for PCR assay were described by Vila *et al.*[68].

*gyrA* F 5'AAATCTGCCCGTGTCGTTGGT3'

*gyrA* R 5'GCCATACCTACGGCGATACC3'

The PCR was performed in 50 µl volumes containing 50 ng of DNA, 25 pM each of primer, 100 µM dNTPs, 2.5 U of *Taq* Pol and PCR buffer (containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2 and 0.001% gelatin). The PCRs were performed under the following conditions. Initial denaturation at 94°C for 10 min, denaturation of 94°C for 1 min for 30 cycles, annealing at 55°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10 min.

**Amplification of *bla* TEM gene**

The oligonucleotide primers used for PCR assay were described by Paterson *et al.*[69].

*bla* TEM F 5' AAACGCTGGTGAAAGTA 3'

*bla* TEM R 5'AGCGATCTGTCTAT3'

The PCR was performed in 50 µl volumes containing 50 ng of DNA, 25 pM each of primer, 100 µM dNTPs, 2.5 U of *Taq* Pol and PCR buffer (containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2 and 0.001% gelatin). PCR conditions included 30 cycles of amplification under the following conditions: denaturation at 95°C for 30 sec, annealing at 45 °C for 1 min at 45°C, and extension at 72°C (for 1 min/kb product). Cycling was followed by a final extension at 72°C for 10 min.

**Amplification of *bla* VIM gene**

The oligonucleotide primers used for PCR assay were described by Miriagou *et al.*[70].

VIM-F (5´-AGTGGTGAGTATCCGACAG-3´)

VIM-R (5´-ATGAAAGTGCGTGGAGAC-3´)

Amplification was performed in a final volume of 50 µL containing 25 picomol of each primer, 25 µM of each dNTP, 1.5 mM $MgCl_2$ and 0.5 µl of *Taq* polymerase (0.05 units/µl). PCR conditions included 30 cycles of amplification under the following conditions: denaturation at 94°C for 30 sec, annealing at 58.2 °C for 1 min and extension at 72°C (for 2 min/kb product). Cycling was followed by a final extension at 72°C for 10 min.

**Sequence analysis of PCR products**

To confirm the amplification of above mentioned genes encoding for resistance, the amplified products were sent to Eurofins Genomics India Pvt. Ltd. (Karnataka) for their sequence analysis.

Five microliters aliquots of PCR products were analyzed by gel electrophoresis with 1% agarose gels (BioRad, Hercules, Calif.) in TAE buffer. Gels were stained with ethidium bromide (10 mg/L) and visualized by Alpha imager EC (Alpha Innotech, USA).

**RESULTS AND DISCUSSION**

**Antibiotic resistance pattern of PTS13**

The isolate selected for this study showed resistance against maximum number of antibiotics i. e. ten different antibiotics which included Amikacin (Aminoglycoside), Amoxiclav (Penicillin combination), Co-trimoxazole (Sulfonamide), Cefotaxime (Cephalosporin), Doxicycline-HCl (Tetracycline), Levofloxacin (Flouroquinolone), Polymixin (Polypeptide), Vancomycin (Glycopeptide), Imipenem & Meropenem (Carbapenems).

**Molecular identification**

The 16rRNA amplified sequence showed maximum similarity of the test strain with *Vibrio alginolyticus.* The sequence has been submitted to NCBI with Accession no. KF443081.

**Biochemical identification of bacteria**

(a) Gram staining: The strain stained pink indicating that it is gram negative and was found to be rod shaped.

(b) Growth on TCBS agar: They produced big yellow colonies.

(c) Glucose fermentation: The color of broth changed to yellow indicating the production of acid from glucose.

(d) Sucrose fermentation: Production of acid was observed.

(e) Lactose fermentation: The strain did not produce acid from Lactose

(f) Hydrogen sulphide ($H_2S$) production: Medium color did not change to black indicating that the strain did not produce $H_2S$.

(g) Bioluminescence: The strain was found to be bioluminescent.

(h) Growth in NaCl: Growth was observed in 0.5%, 1.0% and 1.5% NaCl but there was no growth in 0% NaCl.

(i) Motility agar test/ Stabbing technique method: The culture migrated throughout the medium

**Qualitative and quantitative analysis of genomic DNA**

Quality of genomic DNA was checked on 1% agarose gel (loaded 5 µl) for the single intact band. The gel was run at 110 V for 30 mins. 1 µl of each sample was loaded in Nanodrop 8000 for determining $A_{260/280}$ ratio and 1 µl of each sample was used for determining concentration using Qubit® 2.0 Fluorometer.

**Amplification of specific resistance genes**

In the present study, the types of resistance gene/s located in the chromosome of my test isolate, *Vibrio alginolyticus* strain PTS13 were analyzed. A striking feature of this study is the large number of antibiotic resistance genes found in the isolate. The PCR search revealed that the isolate contained the amplification products for the *bla* CTX-M, *bla* TEM, *gyr*A, *bla* VIM like genes. This suggests that *V. alginolyticus* can be a reservoir for these genes in the aquatic environment.

**Identification of *bla* CTX-M gene**

PCR revealed that the test isolate amplified with *bla* CTX-M gene contained the products for the transcriptional regulator, LysR family with amplicon size ~850 bp (fig. 1).

Nucleotide sequence was submitted in GenBank with Accession no.: KP116294

The LysR-type transcriptional regulator (LTTR) family is a well-characterized group of transcriptional regulators [71, 72]. To date it comprises the largest known family of prokaryotic DNA-binding proteins, with 800 members identified on the basis of their amino acid sequence [73]. After extensive research on LTTR, they are now regarded as global transcriptional regulators, acting as either activators or repressors of single or operonic genes; they are often divergently transcribed but can be located elsewhere on the bacterial chromosome [74, 75].

The LTTRs have evolved a regulatory role over genes with similarly diverse functions, whose products can be involved in drug resistance, for example AmpR LTTR activated the gene function of β-lactamase *Rhodobacter capsulatus* [76] and BlaA LTTR activated the gene function of β-lactamase in *Streptomyces* spp. [77] and could similarly be used as markers for antibiotic-resistant strains [78].

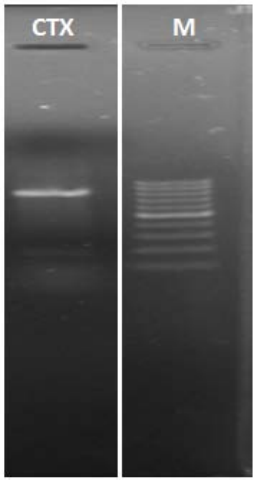


**Fig. 1: 1% Agarose gel shows the gene isolated: Lane1- bla-CTX-M gene ~850 bp and Lane 2- 100 bp Marker**

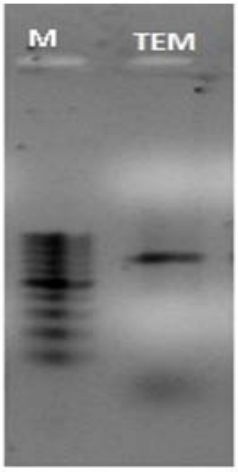


**Fig. 2: 1% Agarose gel shows the gene isolated: Lane1- bla-TEM-M gene ~850 bp and Lane 2- 100 bp Marker**

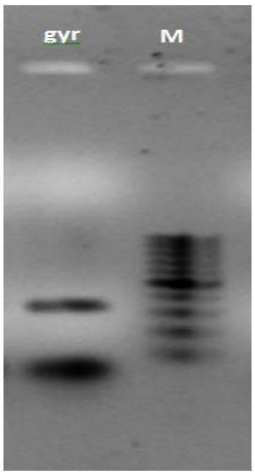


**Fig. 3: 1% Agarose gel shows the gene isolated: Lane1- gyr-A gene ~350 bp and Lane 2- 100 bbp Marker**

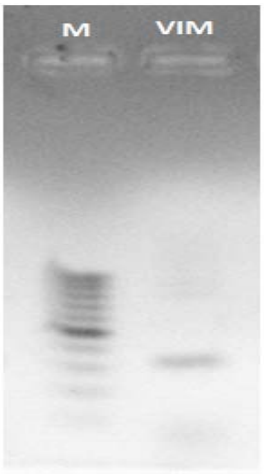


**Fig. 4: 1% Agarose gel shows the gene isolated: Lane1- 100 bp Marker and Lane 2- VIM gene ~350 bp**

Numerous LTTR-regulated antibiotic-resistance factors have been identified as having been acquired by horizontal transfer. The acquisition of antibiotic resistance in *Pseudomonas aeruginosa* is reliant upon the expression of a metallo-β-lactamase, which is regulated by a divergently transcribed LTTR [79]. These LTTRs specifically regulate the genes they are transferred with and do not tend to be global transcriptional regulators [78].

**History of CTX-M**

CTX-M enzymes emerged in the late 1980s, shortly after the introduction of cefotoxime in clinical practice. Since then, wide dissemination of strains carrying these enzymes has occurred, and led to several outbreaks in hospitals and communities [80]. It is associated with multiple drug resistance and is spreading rapidly. The CTX-M type β-lactamases are increasingly found in enterobacterial species throughout the world [14]. The first CTX-M β-lactamase was first isolated in Germany from *E. coli* in 1989 [81], and has rapidly spread into many parts of the world.

The CTX-M type ESBLs are present worldwide with more than 50 variants [31]. The diversity and increasing prevalence of these enzymes pose a serious threat to the clinical use of third-generation cephalosporins for the treatment of severe infections. There is an urgent need to use molecular detection methods that enable the identification and monitoring of the emergence of CTX-M-type ESBLs. PCR has been applied successfully to characterize *bla* CTX-M genes [30, 82, 83]. It has been shown that CTX-M enzymes are not only limited to nosocomial setting but also have a potential for spread beyond the hospital environment [38, 84]. Since 1990s, CTX-M enzymes have become the most prevalent type of ESBLs described around the world in both nosocomial and community settings [40].

**Identification of *bla* TEM gene**

PCR amplification of class A β -lactamase genes revealed that the test isolate contained *bla* TEM gene with amplicon size ~ 850 bp (fig. 2). The nucleotide sequence of *Vibrio alginolyticus* strain PTS13 amplified with *bla* TEM gene exhibited 98% identity with *Vibrio alginolyticus* NBRC 15630 = ATCC 17749 chromosome (fig. 2).

Nucleotide sequence was submitted in Gen Bank with Accession no.: KP116296

**Identification of *gyr*A gene**

The PCR search revealed that the test isolate was amplified with *gyr*A gene with product size ~350 bp (fig. 3). The nucleotide sequence of a fragment of the test strain PTS13 *gyr*A gene exhibited about 98% identity with the *gyr*A gene of *Vibrio alginolyticus* NBRC 15630 = ATCC 17749.

Nucleotide sequence was submitted in Gen Bank with Accession no: KP116295

In several bacteria, mutations conferring quinolone resistance have been found in both *gyr*A and *gyr*B genes of DNA gyrase which are the intracellular target of quinolones. In *Escherichia coli*, several mutations have been identified in the *gyr*A gene [85-87]. Similar mutations have also been identified in quinolone-resistant isolates of *Campylobacter jejuni* [88], and *Pseudomonas aeruginosa* [89].

**Identification of *bla* VIM gene**

PCR screening of the test strain amplified with *bla* VIM revealed the presence of *bla* VIM, with an amplicon of ~350 bp (fig. 4). The amplified sequence of the test isolate showed 98% identity with TolQ protein, proton channel family of *Vibrio alginolyticus* NBRC 15630 = ATCC 17749 chromosome 1.

Nucleotide sequence was submitted to GenBank with Accession no: KP116297

**TolQ protein**

The outer membrane of gram negative bacteria like *Escherichia coli* acts as a permeability barrier against antibiotics, bile salts, and digestive enzymes [90]. However, there are systems which facilitate the uptake of larger molecules. For example, TolQ proteins facilitate the import of both filamentous bacteriophage and group A colicins. The TolQ proteins are involved in the maintenance of cell envelope structure. Mutations in any of the TolQ genes render the cell sensitive to drugs like colicins and induce the release of periplasmic enzymes [91]. TolQ are known for the phenotype of colicin A tolerance [92].

The VIM gene was first found in Europe [45]. It is the most frequent MBLs in America, and they have 12 alelic variants, with the VIM-2 variant the most disseminated worldwide [41]. Metallo beta lactamases including IMP and VIM termed have been found so far in *Acenitobacter. baumannii* and these are encoded by different plasmid types [93]. In 1997, the first enzyme of this family, VIM-1, was found in *P. aeruginosa* in Italy. Many of the eighteen VIM variants identified to date have been reported almost exclusively in *P. aeruginosa.* VIM-2, which shares 90% amino acid identity to that of VIM-1, is the most widespread VIM in terms of both host organism and geography.

Since 2000, the metalloenzymes have shown a remarkable increase worldwide, not only in their prevalence but also in the overall number of enzymes [94, 95]. Surveillance is needed to monitor the spread of MBLs, as this represents an emerging public health threat with implications for antimicrobial use and infection control [96].

Antibiotics can directly affect the rate of emergence of antibacterial resistance determinants in a bacterial population [97]. Lethal doses of antibiotics select for pre-existing resistant strains. Sub-lethal doses can also select for pre-existing resistant strains [98], but in addition, they can favor the emergence of new resistant determinants by increasing the mutation rate and their spread through the stimulation of the horizontal gene transfer [99]. Emergence of resistance through mutations results from the modifications of the antibiotic targets, such as quinolone or rifampicin families, and also for the evolution of genes conferring resistance through enzymatic antibiotic modifications, such as β-lactam and cephalosporins. Mutagenesis is not only induced by antibiotics targeting DNA replication or DNA metabolism, but also by antibiotics that target ribosomes like aminoglycosides or tetracyclines or cell wall synthesis like β-lactam antibiotics [100-103].

## CONCLUSION

Overall, the test isolate PTS13, isolated from the coastal sediment of Tuticorin, have enabled me to identify some important genes responsible for drug resistance. Besides, transcriptional regulators and integral cytoplasmic membrane protein were also identified.

## REFERENCES

1. Kümmerer K. Antibiotics in the aquatic environment-A review-Part I. Chemosphere 2009;75:417-34.
2. Andersson DI, Levin BR. The biological cost of antibiotic resistance. Curr Opin Microbiol 1999;2(5):489-93.
3. Buschmann AH, Tomova A, López A, Maldonado MA, Henríquez LA. Salmon aquaculture and antimicrobial resistance in the marine environment. PLoS One 2012;7(8):1.
4. Goering RV. Mims' Medical Microbiology. 4th ed. Mosby Elsevier; 2008.
5. Bush K. Bench-to-bedside review: The role of β-lactamases in antibiotic-resistant Gram-negative infections. Crit Care 2010;14(3):224.
6. Haldorsen BC, Samuelsen O. Multiresistente Gram-negative bakterier-etøkendeglobalthelse problem. Bioingeniøren; 2012. p. 2.
7. Madigan MT, Martinko JM. Brock biology of microorganisms. Eleventh ed. Pearson Prentice Hall; 2006.
8. Christopher TW, John WT, Patrice C, Julian E, Davies. Mechanism of antibiotic action and resistance. Lancet Infect Diseases; 2001.
9. Biedenbach DJ, Moet GJ, Jones RN. Occurrence and antimicrobial resistance pattern comparisons among bloodstream infection isolates from the SENTRY Antimicrobial Surveillance Program (1997-2002). Diagn Microbiol Infect 2004;50(1):59-69.
10. National nosocomial infection surveillance (NNIS) system report. Data summary from January 1992 through June 2004, issued October 2004. Am J Infect Control 2004;32:470-85.
11. Pan XS, Gould KA, Fisher LM. Probing the differential interactions of quinazolinedione PD 0305970 and quinolones with gyrase and topoisomerase IV. Antimicrob Agents Chemother 2009;53(9):3822–31.
12. Acar JF, Goldstein FW. Trends in bacterial resistance to fluoroquinolones. Clin Infect Dis 1997;24(1):67-73.
13. Jalal KCA, Akbar John B, Kamaruzzaman BY, Kathiresan K. Antibiotic resistant bacteria from coastal environment-a review. In: Marina Pana ed. Antibiotic resistant bacteria-a continuous challenge in the new millennium. Rijeka, In Tech; 2012. p. 143-58.
14. Bonnet R. Growing group of extended-spectrum beta-lactamases: the CTX-M enzymes. Antimicrob Agents Chemother 2004;48:1–14.
15. Poole K. Resistance to β-lactam antibiotics. Cell Mol Life Sci 2004;61:2200–23.
16. Jacoby GA. AmpC β-lactamases. Clin Microbiol Rev 2009;22(1):161-82.
17. Bush K, Jacoby GA. Updated functional classification of β-lactamases. Antimicrob Agents Chemother 2010;54(3):969-76.
18. Hall BG, Barlow M. Revised ambler classification of β-lactamases. J Antimicrob Chemother 2005;55(6):1050-1.
19. Livermore DM. β-Lactamases in laboratory and clinical resistance. Clin Microbiol Rev 1995;8(4):557-84.
20. Datta N, Kontomichalou P. Penicillinase synthesis controlled by infectious R factors in Enterobacteriaceae. Nat 196;208:239–41.
21. Hiroshi Nikaido. Multidrug resistance in bacteria. Annu Rev Biochem 2009;78:119–46.
22. Bradford PA. Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin Microbiol Rev 2001;14(4):933-51.
23. Matthew M, Hedges RW, Smith JT. Types of beta-lactamase determined by plasmids in Gram-negative bacteria. J Bacteriol 1979;138:657–62.
24. Kliebe C, Nies BA, Meyer JF, Tolxdorff-Neutzling RM, Wiedemann B. Evolution of plasmid-coded resistance to broad-spectrum cephalosporins. Antimicrob Agents Chemother 1985;288:302–7.
25. Podbielski A, Schonling J, Melzer B, Warnatz K, Leusch HG. Molecular characterization of a new plasmid-encoded SHV-type beta-lactamase (SHV-2 variant) conferring high-level cefotaxime resistance upon *Klebsiellapneumoniae*. J Gen Microbiol 1991;137:569–78.
26. Sirot D, Sirot J, Labia R. Transferable resistance to third-generation cephalosporins in clinical isolates of *Klebsiellapneumoniae*: identification of CTX-1, a novel beta-lactamase. J Antimicrob Chemother 1987;20:323–34.
27. Sougakoff W, Goussard S, Courvalin P. The TEM-3 beta-lactamase, which hydrolyzes broad-spectrum cephalosporins, is derived from the TEM-2 penicillinase by two amino acid substitutions. FEMS Microbiol Lett 1988;56:343–8.
28. Quale JM, Landman D, Bradford PA, Visalli M, Ravishankar J, Flores, *et al.* Molecular epidemiology of a citywide outbreak of extended-spectrum β-lactamase producing *Klebsiellapneumoniae* infections. Clin Infect Dis 2002;35:834-41.
29. Paterson DL, Ko WC, A Von Gottberg, Mohapatra S, JM Casellas, Goossens H, *et al.* Implications of extended-spectrum beta-lactamase production in nosocomial infections international prospective study of Klebsiella pneumonia bacteremia. Ann Intern Med 2004;140:26-32.
30. Pitout JD, Hossain A, Hanson ND. Phenotypic and molecular detection of CTX-M-beta-lactamases produced by *Escherichia coli* and Klebsiella spp. J Clin Microbiol 2004;42:5715–21.
31. Perez F, Endimiani A, Hujer KM, Bonomo RA. The continuing challenge of ESBLs. Curr Opin Pharmacol 2007;7(5):459–69.
32. Tzouvelekis LS, Tzelepi E, Tassios PT, Legakis NJ. CTX-M-type β-lactamases: an emerging group of extended-spectrum enzymes. Int J Antimicrob Agents 2000;14:137-42.
33. Barlow M, Reik RA, Jacobs SD, Medina M, Meyer MP. High rate of mobilization for blaCTX-M. Emerg Infect 2008;14:423–8.
34. Canton R, Coque TM. The CTX-M beta-lactamases pandemic. Curr Opin Microbiol 2006;9:466-75.
35. Canton R, Novais A, Valverde A, Machado E, Peixe L, Baquero F, *et al*. Prevalence and spread of extended-spectrum beta-lactamase-producing Enterobacteriaceae in Europe. Clin Microbiol Infect 2008;14:144-53.
36. Hawkey PM. Prevalence and clonality of extended-spectrum beta-lactamases in Asia. Clin Microbiol Infect 2008;14:159-65.
37. Peleg AY, Hooper DC. Hospital-acquired infections due to Gram-negative bacteria. N Engl J Med 2010;362:1804-13.
38. Pitout JD, Laupland KB, Poirel L. Emergence of Enterobacteriaceae producing extended-spectrum beta-lactamase (ESBLs n the community. J Antimicrob Chemother 2005;56:52-9.
39. Rossolini GM, D'Andrea MM, Mugnaioli C. The spread of CTX-M-type extended-spectrum beta-lactamases. Clin Microbiol Infect 2008;14:33-41.

40. Villegas MV, Kattan JN, Quinoteros MG, Casellas JM. Prevalence of extended-spectrum beta-lactamases in South America. Clin Microbiol Infect 2008;14:154-8.
41. Sánchez D, Marcano D, Spadola E, León L, Payares P, Ugarte C, *et al*. Metaloenzimastipo VIM detectadas en aislamientos clínicos en pseudomonas aeruginosaen Cuatro Hospitalesen Venezuela. Revista Científicadel Instituto Nacional de Higiene "Rafael Rangel"; 2008.
42. Toleman M, Biedenbach D, Bennett D, Jones R, Walsh T. Italian metallo-lactamases: a national problem? Report from the sentry antimicrobial surveillance programme. J Antimicrob Chemother 2005;55:61-70.
43. Sader H, Castanheira M, Mendes R, Toleman M, Walsh T, Jones R. Dissemination and diversity of metalo b-lactamases in Latin America: report from the sentry antimicrobial surveillance program. Int J Antimic Agents 2005;25:57-61.
44. Peleg A, Franklin C, Bell J, Spelmam D. Dissemination of the metallo β-lactamase gene bla IMP-4 among gram-negative pathogens in a clinical setting in Australia Clin Infect Dis 2005;41:1549-56. Walsch TR, Toleman MA, Poirel L, Nordmann P. Metallo-beta-lactamases: the quiet before the storm? Clin Microbiol Rev 2005;18:306-25.
45. Walsch TR, Toleman MA, Poirel L, Nordmann P. Metallo-beta-lactamases: the quiet before the storm? Clin Microbiol Rev 2005;18:306-25.
46. Pitout JDD, Chow BL, Gregson DB, Laupland KB, Elsayed S, Church DL. Molecular epidemiology of metallo-β-lactamase-producing *Pseudomonas aeruginosa* in the Calgary Health Region: emergence of VIM-2-producing isolates. J Clin Microbiol 2007;45(2):294–8.
47. Khosravi Y, Tay ST, Vadivelu J. Analysis of integrons and associated gene cassettes of metallo-β-lactamase-positive *Pseudomonas aeruginosa* in Malaysia. J Med Microbiol 2011;60(7):988–94.
48. Ellington MJ, Kistler J, Livermore DM, Woodford N. Multiplex PCR for rapid detection of genes encoding acquired metallo-β-lactamases. J Antimicrobial Chemother 2007;59(2):321–2.
49. Giakkoupi P, Tzouvelekis L, Daikos G, Miriagou V, PetrikkosM G, Legakis N, *et al*. Discrepancies and interpretation problems in susceptibility testing of VIM-1-producing *Klebsiellapneumoniae* isolates. J Clin Microbiol 2005;43(1):494-6.
50. Conceicâo T, Brizio A, Duarte A. First isolation of blaVIM-2 in *Klebsiellaoxytoca* clinical isolates from portugal. Antimicrobial Agents Chemother 2005;49(1):476.
51. Drlica K, Hiasa H, Kerns R, Malik M, Mustaev A, Zhao X. Quinolones: action and resistance updated. Top Med Chem 2009;9:981–98.
52. Fabrega A, Sanchez-Cespedes J, Soto S, Vila J. Quinolone resistance in the food chain. Int J Antimicrob Agents 2008;31:307–15.
53. Drlica K, Zao X. DNA gyrase, topoisomerase IV, and the 4-quinolones. Mol Biol Rev 1997;61:377–92.
54. Malik M, Marks KR, Mustaev A, Zhao XL, Chavda K, Kerns RJ, *et al*. Fluoroquinolone and quinazolinedione activities against wild-type and gyrase mutant strains of mycobacterium smegmatis. Antimicrob Agents Chemother 2011;55:2335–43.
55. Lin, XM, Li H, Wang C, Peng XX. Proteomic analysis of nalidixic acid resistance in Escherichia coli: identification and functional characterization of OM proteins. Proteome Res 2008;7(6):2399–405.
56. Ruiz J. Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection. J Antimicrob Chemother 2003;51(5):1109-17.
57. Robicsek A. Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. Nat Med 2006;12(1):83-8.
58. Biedenbach DJ, Jones RN. Fluoroquinolone-resistant *Haemophilusinfluenzae*: frequency of occurrence and analysis of confirmed strains in the sentry antimicrobial surveillance program (North and Latin America). Diagn Microbiol Infect 2000;36:255–9.
59. Gales AC, Gordon KA, Wilke WW, Pfaller MA, Jones RN. Occurrence of single-point *gyr*A mutations among ciprofloxacin-susceptible Escherichia *coli* isolates causing urinary tract infections in Latin America. Diagn Microbiol Infect Dis 2000;36:61–4.
60. Harriague A, Covazzi, Brino MD, Zampini M, Albertelli G, Pruzzo C, *et al*. Vibrios in association with sedimentary crustaceans in three beaches of the northern Adriatic Sea (Italy). Mar Pollut Bull 2008;56:574-9.
61. Ben Kahla-Nakbia A, Besbesa A, Chaieba K, Rouabhiab M, Bakhroufa A. Survival of *Vibrio alginolyticus* in sea water and retention of virulence of its starved cells. Mar Environ Res 2007;46:469-78.
62. Rheinheimer G. Microbial ecology of brakish water environment. Ecol Stud 1977;28:39-60.
63. Bauer AW, Kirby WM, Sherris JC, Jurck M. Antibiotic susceptibility testing by a standard single disc method. Am J Clin Pathol 1996;451:493-6.
64. Sambrook J, Ressell DW. Molecular cloning-A laboratory manual. Cold Spring Harbor Laboratory, 3rd edition. Cold Spring Harbor: New York; 2001.
65. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, *et al*. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Sci 1988;239:487-91.
66. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study". J Bacteriol 1991;173(2):697–703.
67. Yang-Ree Kim, Sang-Il Kim, Ji-Young Lee, Y eon-Joon Park, Kyo-Young Lee, Moon-Won Kang. Nosocomial transmission of CTX-M-15 and OXA-30 β-Lactamase-producing *Escherichia coli* in a neurosurgical intensive care unit. Ann Clin Lab Sci 2005;35:3.
68. Vila J, Ruiz P, Goni A, Marcos, Jimenez de Anta T. Mutation in the *gyr*A gene of quinolone-resistant clinical isolates of *Acinetobacter baumannii*. Antimicrob Agents Chemother 1995;39:1201–3.
69. Paterson DL, Hujer KM, Hujer AM, Yeiser B, Bonomo MD, L Rice LB, *et al*. Extended-spectrum lactamases in *Klebsiella pneumonia* bloodstream isolates from seven countries: dominance and widespread prevalence of SHV-and CTX-M-type-lactamases. Antimicrob Agents Chemother 2003;47:3554–60.
70. Miriagou V, Tzelepi E, Gianneli D, Tzouvelekis LS. *Escherichia coli* with a self-transferable, multiresistant plasmid coding for metallo-β-lactamase VIM-1. Antimicrob Agents Chemother 2003;47:395–7.
71. Sun J, Klein A. A LysR-type regulator is involved in the negative regulation of genes encoding selenium-free hydrogenases in the archaeon *Methanococcus voltae*. Mol Microbio 2004;l52:563–71.
72. Stec E, Witkowska-Zimny M, Hryniewicz MM, Neumann P, Wilkinson AJ, Brzozowski AM, *et al*. Structural basis of the sulphate starvation response in E. coli: crystal structure and mutational analysis of the cofactor-binding domain of the Cbl transcriptional regulator. J Mol Bio 2006;l364:309–22.
73. Schell MA. Molecular biology of the LysR family of transcriptional regulators. Annu Rev Microbio 1993;l47:597–626.
74. Heroven AK, Dersch P. Rov M, a novel Lys R-type regulator of the virulence activator generovA, controls cell invasion, virulence and motility of *Yersinia pseudotuberculosis*. Mol Microbiol 2006;62:1469–83.
75. Herna´ndez-Lucas I, Gallego-Herna´ndez AL, Encarnacio´n S, Ferna´ndez-Mora M, Martı´nez-Batallar AG, Salgado H, *et al*. The Lys R-type transcriptional regulator LeuO controls expression of several genes in *Salmonella enterica* serovar Typhi. J Bacteriol 2008;190:1658–70.
76. Bartowsky E. Normark S. Interactions of wild-type and mutant AmpR ofCitrobacter freundiiwith target DNA. Mol Microbiol 1993;10:555–65.
77. Raskin C, Ge´rard C, Donfut S, Giannotta E, Van Driessche G, Van Beeumen J, Dusart J. BlaB, a protein involved in the

regulation of *Streptomyces cacaoi* β-lactamases, is a penicillin-binding protein. Cell Mol Life Sci 2003;60:1460–9.
78. Sarah E Maddocks, Petra CF. Structure and function of the LysR-type transcriptional regulator (LTTR) family protein. Rev Oyston Microbiol 2008;154:3609–23.
79. Toleman MA, Simm AM, Murphy TA, Gales AC, Biedenbach DJ, Jones RN, *et al*. Molecular characterization of SPM-1, a novel metallo-b-lactamase isolated in Latin America: report from the SENTRY antimicrobial surveillance programme. J Antimicrob Chemother 2002;50:673–9.
80. Arlet G, Barrett TJ, Butaye P, Cloeckaert A, Mulvey MR, White DG. Salmonella resistance to extended-spectrum cephalosporins: prevalence and epidemiology. Microbes Infect 2006;8:1945–54.
81. Bauernfeind A, Grimm H, Schweighart S. A new plasmidic cefotaximase in a clinical isolate of *Escherichia coli*. Infect 1990;18:743-7.
82. Canton R, Oliver A, Coque TM, Varela MC, Perez-Diaz JC, Baquero F. Epidemiology of extended-spectrum beta-lactamase-producing Enterobacter isolates in a Spanish hospital during a 12-year period. J Clin Microbiol 2002;l40:1237–43.
83. Chanawong A, M'Zali FH, Heritage J, Xiong JH, Hawkey PM. Three cefotaximases, CTX-M-9, CTX-M-13, and CTX-M-14, among Enterobacteriaceae in the people's republic of china. Antimicrob Agents Chemother 2002;46:630–7.
84. Pitout JD, Laupland KB. Extended-spectrum-beta-lactamse-producing Enterobacteriaceae: an emerging public-health concern. Lancet Infect Dis 2008;8:159-66.
85. Cambau E, Bordon F, Collatz E, Gutmann L. Novel *gyr*A point mutation in a strain of *Escherichia coli* resistant to fluoroquinolones but not to nalidixic acid. Antimicrob Agents Chemother 1993;37:1247–52.
86. Heisig P, Schedletzky H, Falkenstein-Paul H. Mutations in gyrA gene of a highly fluoroquinolone-resistant clinical isolate of *Escherichia coli*. Antimicrob Agents Chemother 1993;37:696–701.
87. Vila J, Ruiz J, Marco F, Barcelo A, Goni P, Giralt E, Jimenez De Anta T. Association between double mutation in *gyr*A gene of ciprofloxacin-resistant clinical isolates of *Escherichia coli* and MICs. Antimicrob. Agents Chemother 1994;38:2477–9.
88. Wang Y, Huang WM, Taylor DE. Cloning and nucleotide sequence of the Campylobacter *jejuni gyr*A gene and characterization of quinolone resistance mutations. Antimicrob Agents Chemother 1993;37:457–63.
89. Kureishi A, Diver JM, Beckthold B, Schollaardt T, Bryan LE. Cloning and nucleotide sequence of *Pseudomonas aeruginosa* DNA gyrase *gyr*A-gene from strain PAO1 and quinolone-resistant clinical isolates. Antimicrob Agents Chemother 1994;38:1944–52.
90. Nikaido H, Vaara M. Molecular basis of bacterial outer membrane permeability. Microbiol Rev 1985;49:1-32.
91. Lazzaroni JC, Portalier R. The excC gene of *Escherichia coli* K-12 required for cell envelope integrity encodes the peptidoglycan-associated lipoprotein. Mol Microbiol 1992;6:735-42.
92. Anne V, Tal ML, Wayne FB, Jean CL, Raymond P, Robert EW. Membrane topology and mutational analysis of the TolQ protein of *Escherichia coli* required for the uptake of macromolecules and cell envelope. Integrity J Bacteriol 1994;176(3):822-9.
93. Walsch TR, Toleman MA, Poirel L, Nordmann P. Metallo-β-Lactamases: the Quiet before the Storm? J Clin Microbiol 2002;18:3798–801.
94. Jones RN, Biedenbach DJ, Sader HS. Emerging epidemic of metallo-beta-lactamase-mediated resistances. Diagn Microbiol Infect Dis 2005;51:77–84.
95. Fritsche TR, Sader HS, Toleman MA. Emerging metallo-beta-lactamase-mediated resistances: a summary report from the worldwide SENTRY antimicrobial surveillance program. Clin Infect Dis 2005;41(4):S276–8.
96. Cornaglia G, Akova M, Amicosante G. Metallo-beta-lactamases as emerging resistance determinants in Gram-negative pathogens: open issues. Int J Antimicrob Agents 2007;29(4):380-8.
97. Koskiniemi S, Pranting M, Gullberg E, Nasvall J, Andersson DI. Activation of cryptic aminoglycoside resistance in *Salmonella enterica*. Mol Microbiol 2011;80:1464 –78.
98. Gullberg E, Cao S, Berg OG, Ilback C, Sandegren L, Hughes D, *et al*. Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathog 2011;7:e1002-158.
99. Blazquez J, Couce A, Rodriguez-Beltran J, Rodriguez-Rojas A. Antimicrobials as promoters of genetic variation. Curr Opin Microbiol 2012;15:561–9.
100. Baharoglu Z, Mazel D. *Vibrio cholerae* triggers SOS and mutagenesis in response to a wide range of antibiotics: A route towards multiresistance. Antimicrob Agents Chemother 2011;55:2438–41.
101. Gutierrez A, Laureti L, Crussard S, Abida H, Rodriguez Rojas A, Blazquez J, *et al*. J Antimicrob Chemother 2011;66:539–42.
102. Thi TD, Lopez E, Rodriguez-Rojas A, Rodriguez-Beltran J, Couce A, Guelfo JR, Castaneda-Garcia A, *et al*. Effect of recA inactivation on mutagenesis of *Escherichia coli* exposed to sublethal concentrations of antimicrobials. J Antimicrob Chemother 2011;66:531–8.
103. Kohanski MA, DePristo MA, Collins JJ. Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. Mol Cell 2010;37:311–20.