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OMICS Group International is an amalgamation of Open Access publications and worldwide international science conferences and events. Established in the year 2007 with the sole aim of making the information on Sciences and technology 'Open Access', OMICS Group publishes 400 online open access scholarly journals in all aspects of Science, Engineering, Management and Technology journals. OMICS Group has been instrumental in taking the knowledge on Science & technology to the doorsteps of ordinary men and women. Research Scholars, Students, Libraries, Educational Institutions, Research centers and the industry are main stakeholders that benefitted greatly from this knowledge dissemination. OMICS Group also organizes 300 International conferences annually across the globe, where knowledge transfer takes place through debates, round table discussions, poster presentations, workshops, symposia and exhibitions.

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OMICS Group International is a pioneer and leading science event organizer, which publishes around 400 open access journals and conducts over 300 Medical, Clinical, Engineering, Life Sciences, Pharma scientific conferences all over the globe annually with the support of more than 1000 scientific associations and 30,000 editorial board members and 3.5 million followers to its credit.

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Inhibitors against Resistant markers: a Molecular and computational Biology approach

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My 20 Years Research journey

Antibiotic Resistance: A Global Concern

- Throughout history there has been a continual battle between human beings and multitude of micro-organisms that cause infection and disease

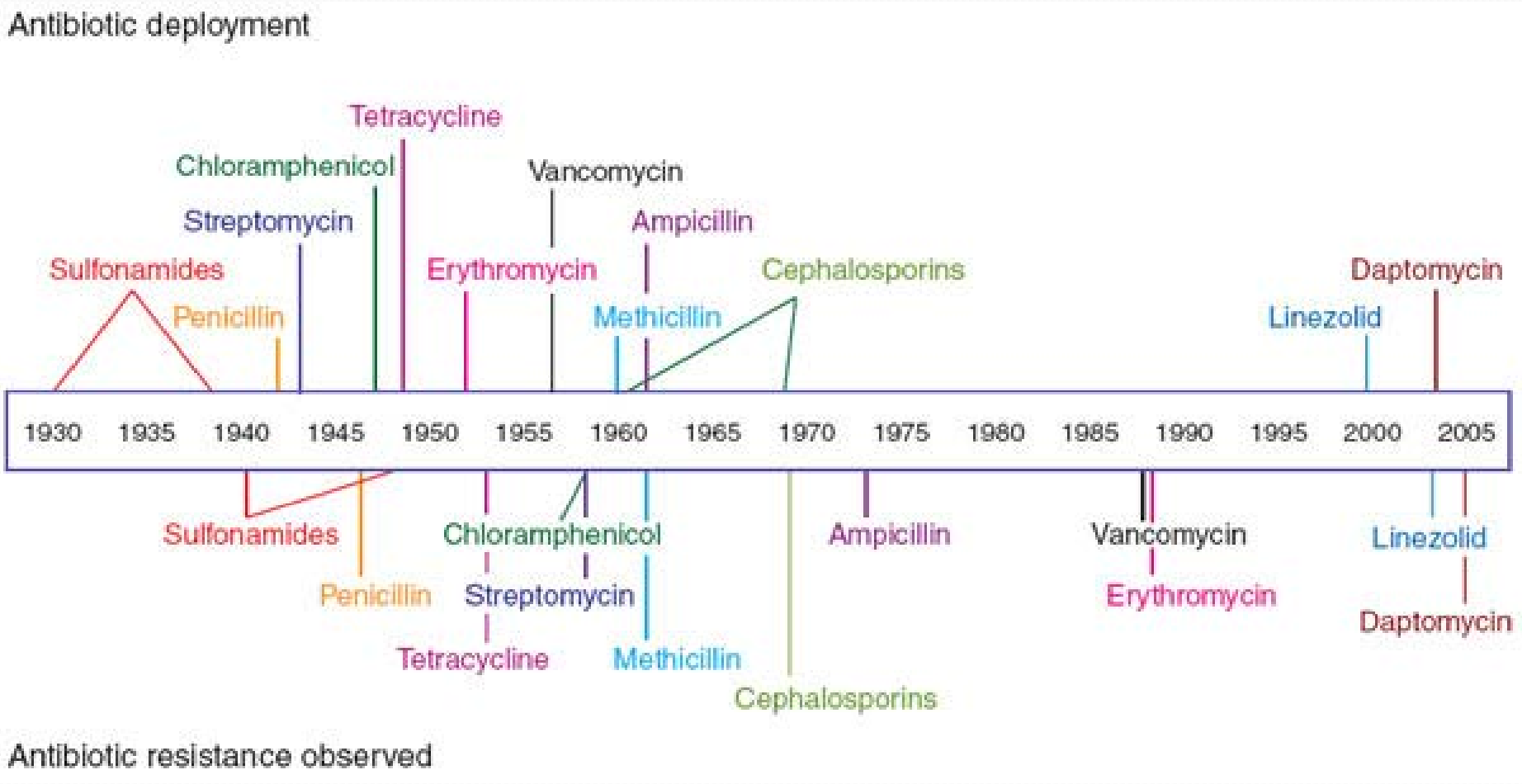
- Triumph of mankind over disease causing bacteria with antibiotics did not last long and soon bacteria fought back demonstrating their remarkable ability to evolve different mechanisms to resist the action of antibiotics

- Antibiotic resistance occurs when an antibiotic has lost its ability to effectively control or kill bacterial growth; in other words, the bacteria are "resistant" and continue to multiply in the presence of therapeutic levels of an antibiotic.

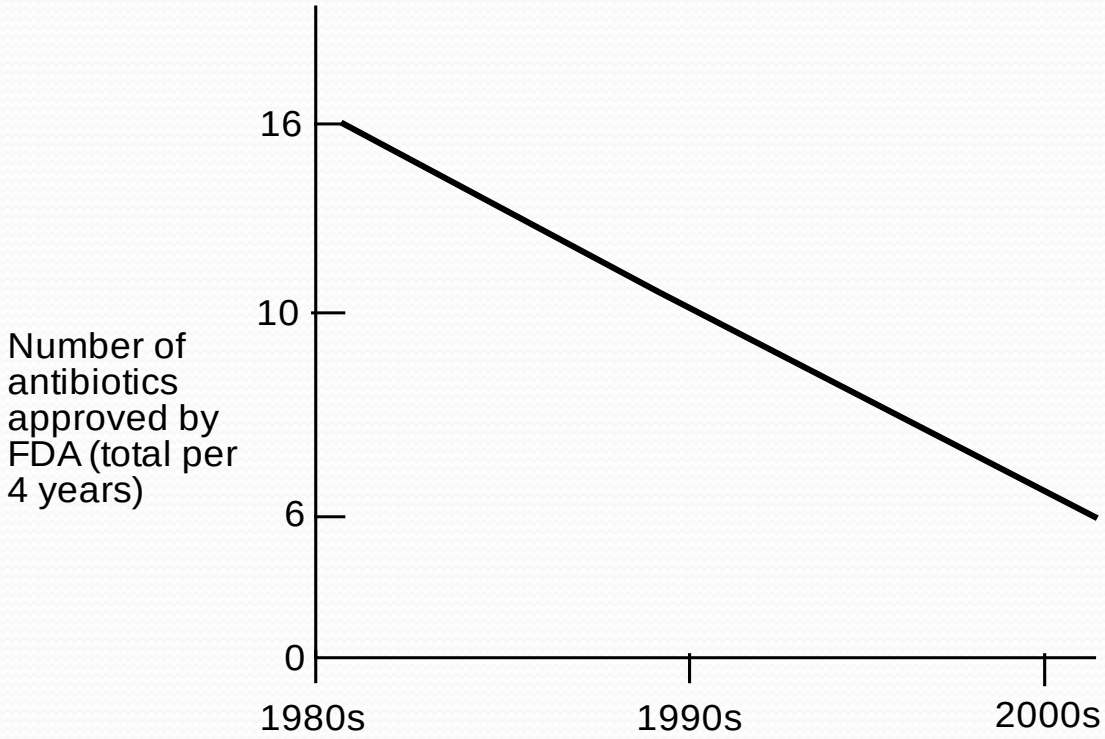
Why Resistance is a Concern?

- Resistant organisms lead to treatment failure.
- Increased mortality
- Added burden on healthcare costs
- Resistant bacteria may spread in the community
- Threatens failure of current antibiotics in use and return to pre-historic era.

Timeline of Antibiotic Resistance



The number of new antibiotics which reach the market is falling



Mechanisms of Resistance

Intrinsic (Natural)
e.g. Lack of cell wall,
Innate efflux pumps etc.

Acquired

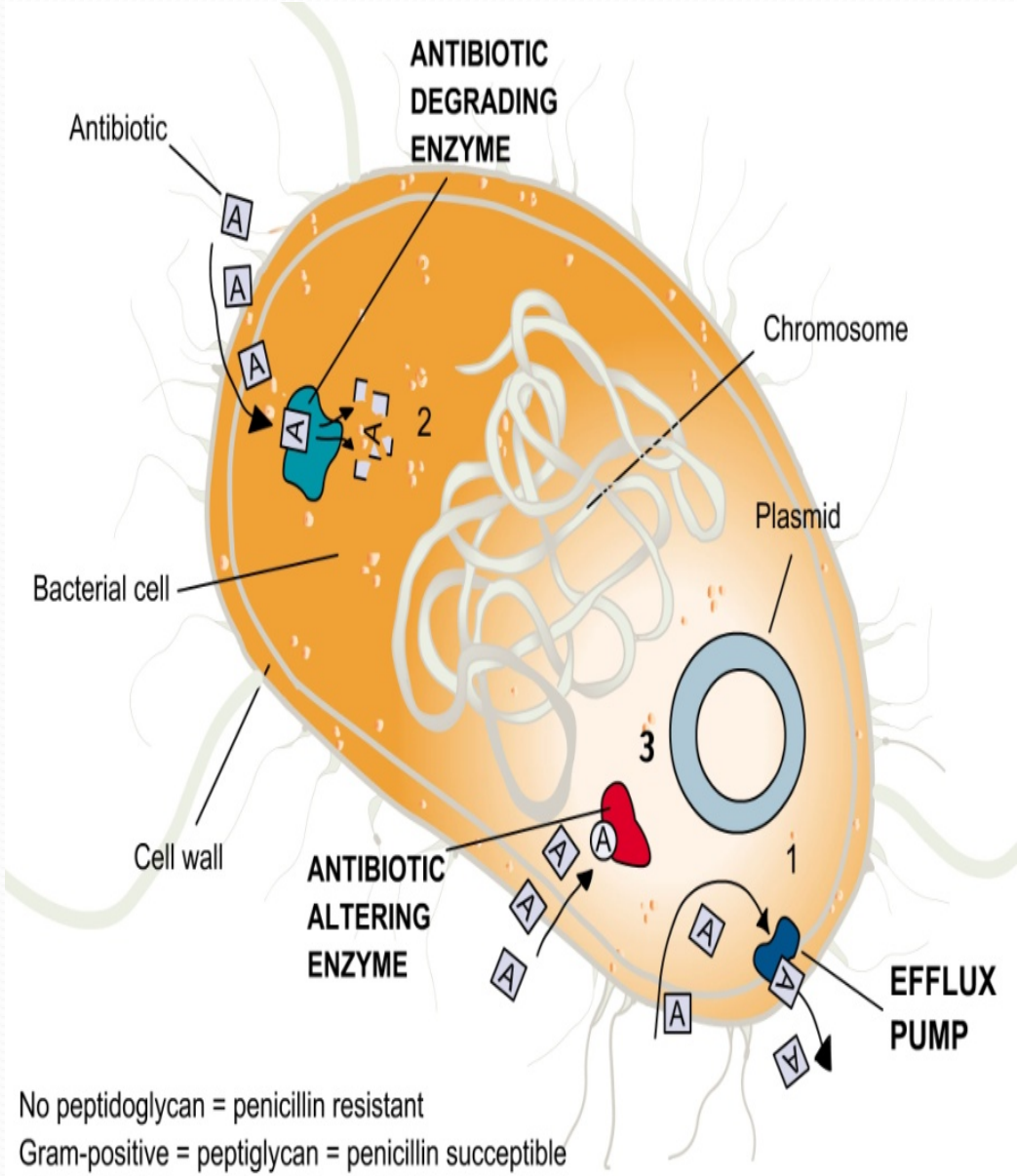
Genetic Methods

<u>Chromosomal Methods</u>
Mutations

<u>Extra chromosomal Methods</u>
Plasmids
(Conjugation, transduction,
Transformation)
(Transposons, Integrons)

Biochemical Mechanisms of Resistance

- By producing enyme that can inactivate the drug
- Prevention of drug accumulation at the target site
- Modification of the active/target site
- Use of alternative pathways for metabolic / growth requirements

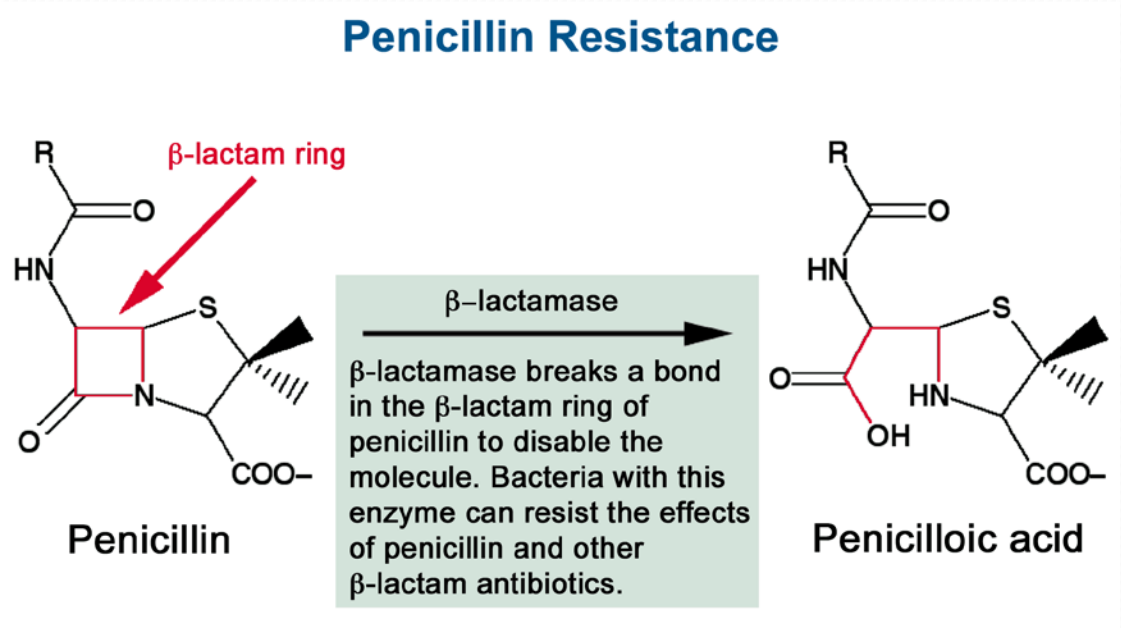


Enzyme-Mediated Resistance: Beta-lactamases

- β-lactamases are enzymes that hydrolyze the β-lactam ring present in β-lactam group of antibiotics like penicillins, cephalosporins and carbapenems

- Without the β-lactam ring, antibiotics are inactive.

- Production of β-lactamase is most common mechanism of resistance in Gram negative bacteria



Classification of β-Lactamases

Molecular class	Functional group	Beta-lactamases
C	1	AmpC
A	2b	TEM-1, TEM-2, SHV-1
	2be	TEM-3, SHV-2, CTX-M-15
	2br	TEM-30, SHV-10
	2ber	TEM-50
D	2d	OXA-1
	2de	OXA-11
	2df	OXA-23
	2f	KPC-2
B	3a	IMP-1, VIM-1

The fight goes on…

Beta-lactams

Beta-lactamases

Beta-lactamase inhibitors

Inhibitor resistant beta-lactamases

Emerging Resistance to Classical Inhibitors

Three Classical Inhibitors

- Clavulanic acid
- Sulbactam
- Tazobactam

Resistance to inhibitors may arise from

- Production of β-lactamase enzymes not susceptible to inhibitors
- Hyper production of β-lacatamases
- Modification of outer membrane permeability

•Cloning and Expression KPC-2 β-lactamase.

•Optimization of expression of the soluble KPC-2 protein by IPTG.

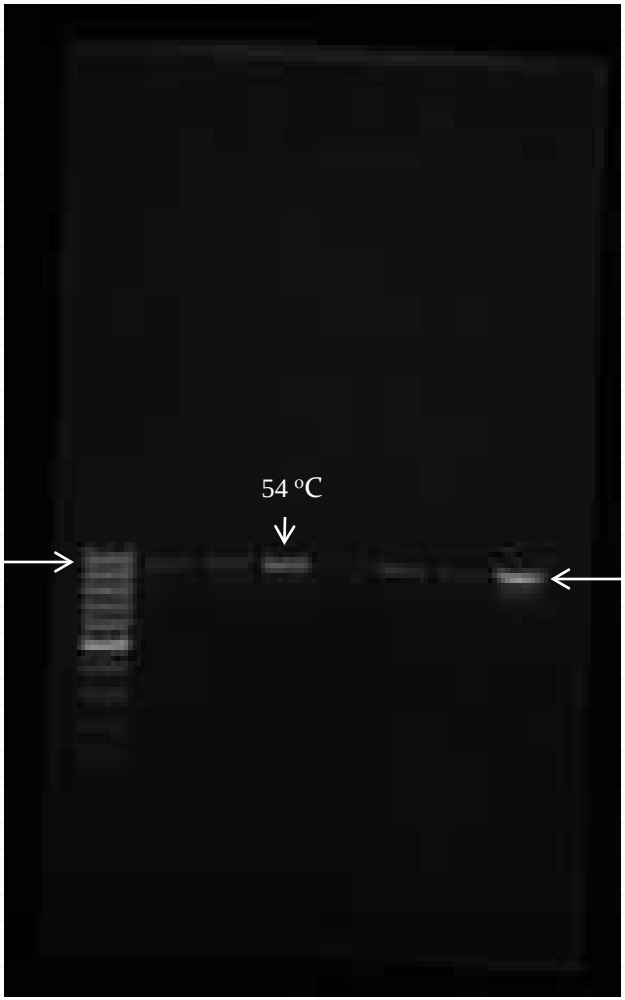
•Purification of recombinant KPC-2 protein

▪ Designing of novel non beta-lactam inhibitors against KPC-2.

▪ Evaluation of efficiency of novel inhibitors in vitro on bacterial cells and on purified enzyme KPC-2.

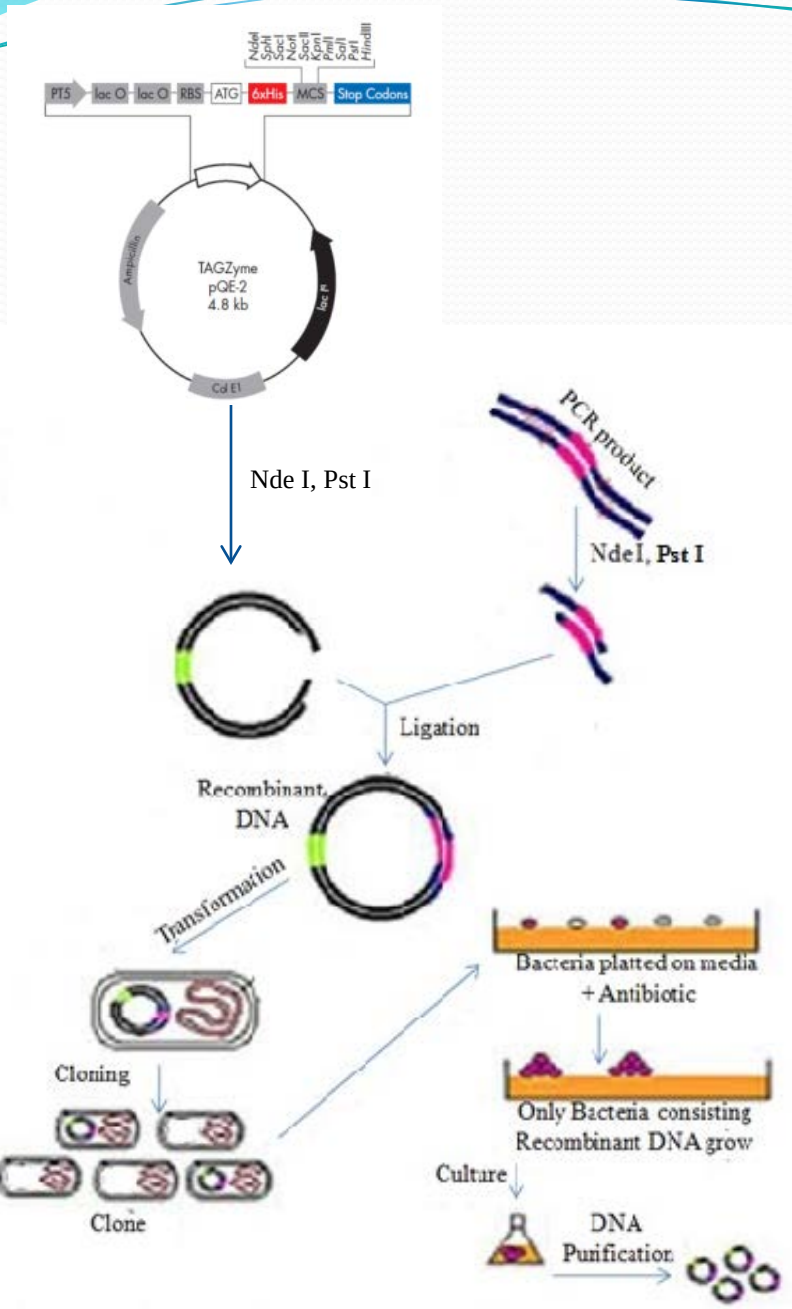
•Whole cell proteome study of carbapenem resistant clinical (NP6) *Klebsiella pneumoniae* in presence and absence of meropenem.

Standardization of PCR conditions for optimal amplification



Standardization of PCR conditions for amplification of *bla*$_{KPC-2}$ from *K. pneumoniae* clinical isolate. Agarose gel showing amplified PCR product at annealing temperatures ranging from 52°C to 58°C in lanes 2 to 8 respectively. Lane 1 contains DNA ladder (100bp). Optimal amplification was at 54°C.

Cloning Procedure



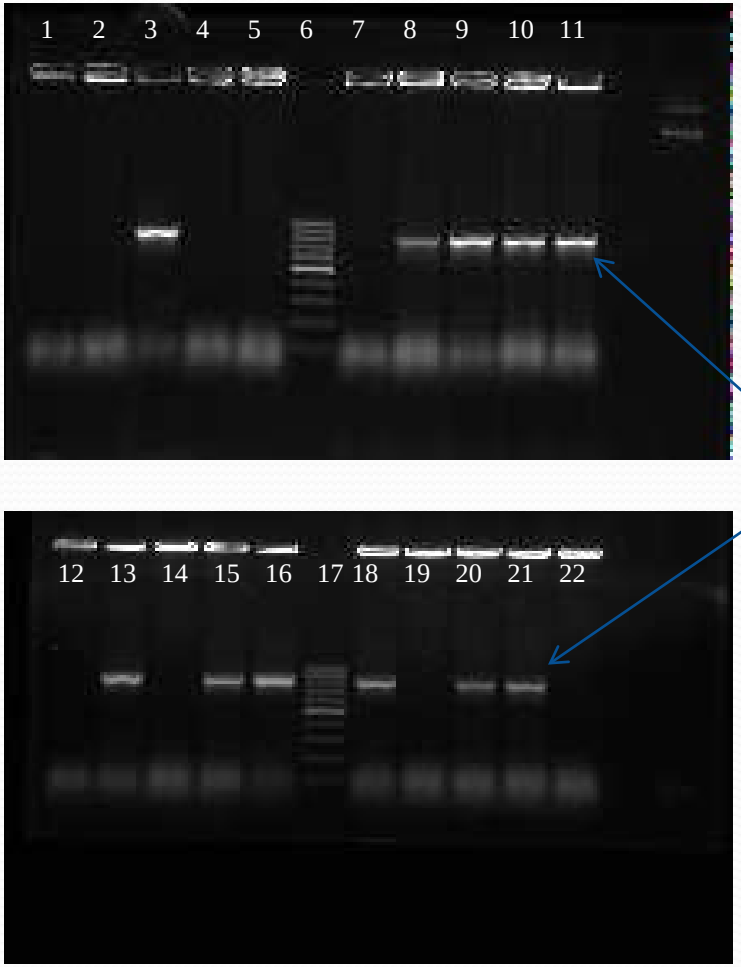
Clones containing plasmid

Confirmation of Positive Clones

The successful clones of *bla*$_{\text{KPC-2}}$ were confirmed through

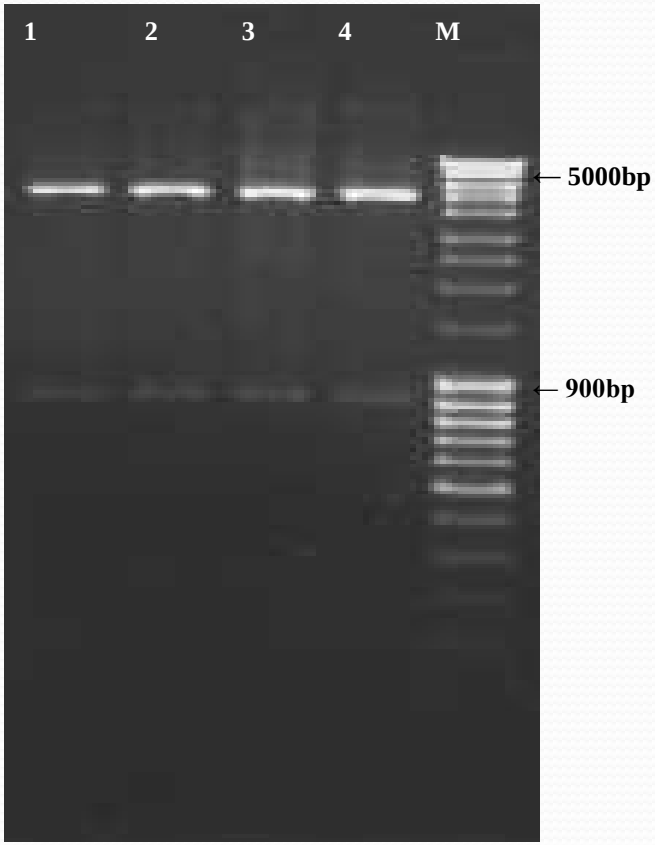
- Colony PCR
- Double digestion method
- Sequencing

Colony PCR



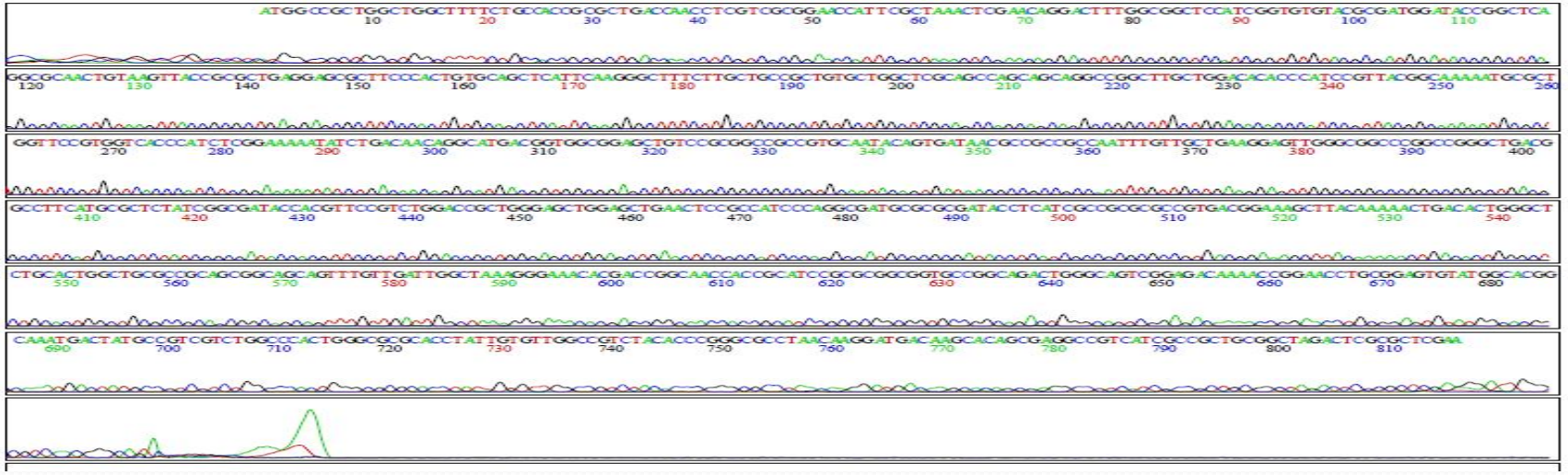
Agarose gel showing amplified gene product from colony PCR. Lane 3, 8, 9, 10, 11, 13, 15, 16, 18, 20, 21 contains positive clones harbouring $bla_{\text{KPC-2}}$ gene. Lane 6 and 17 contain DNA ladder (100bp).

Double Digestion by Nde I and Pst I

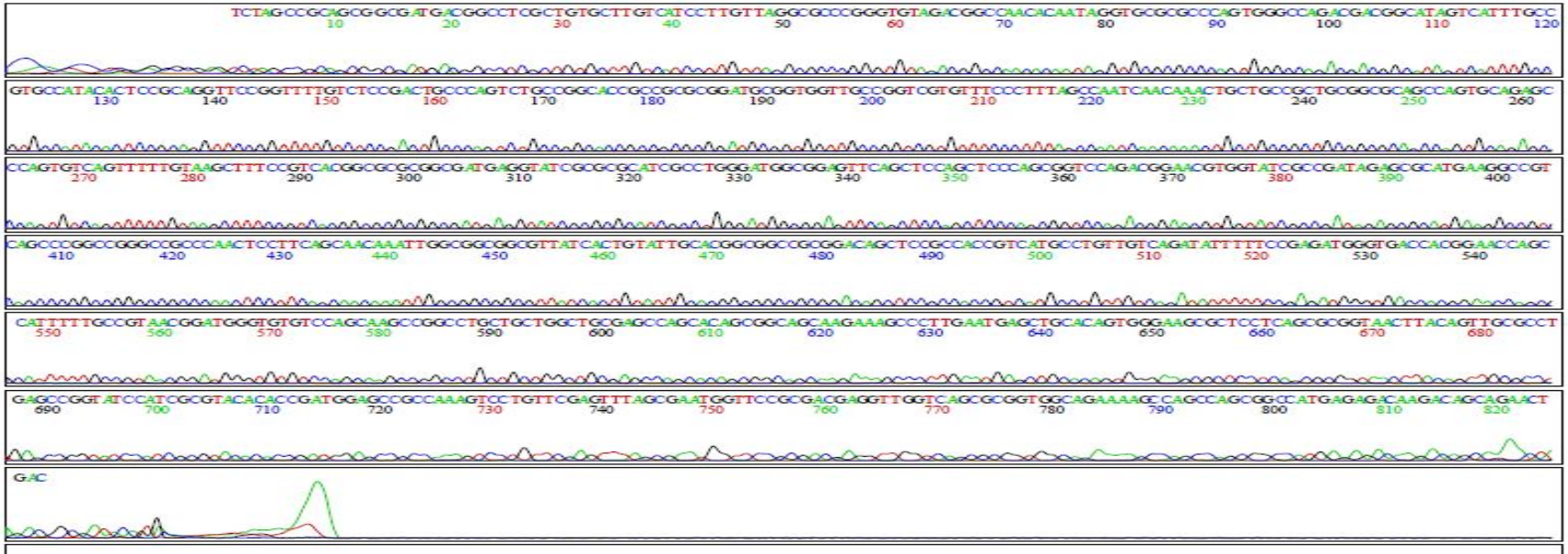


Agarose gel showing vector pQE-2 harbouring *bla*$_{\text{KPC-2}}$ digestion. Larger vector backbone fragment of approx. 5000bp and CTX-M-15 gene at 900bp.

Sequencing

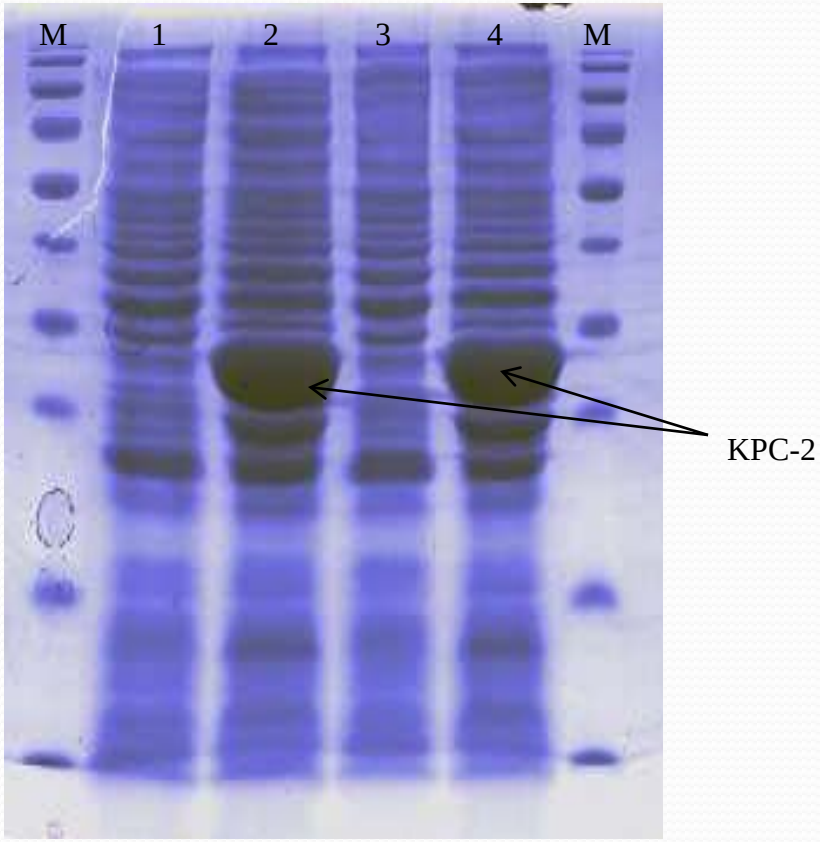


KPC-F



KPC-R

Expression of Recombinant KPC-2 Protein in Transformed E. coli BL21 Cells



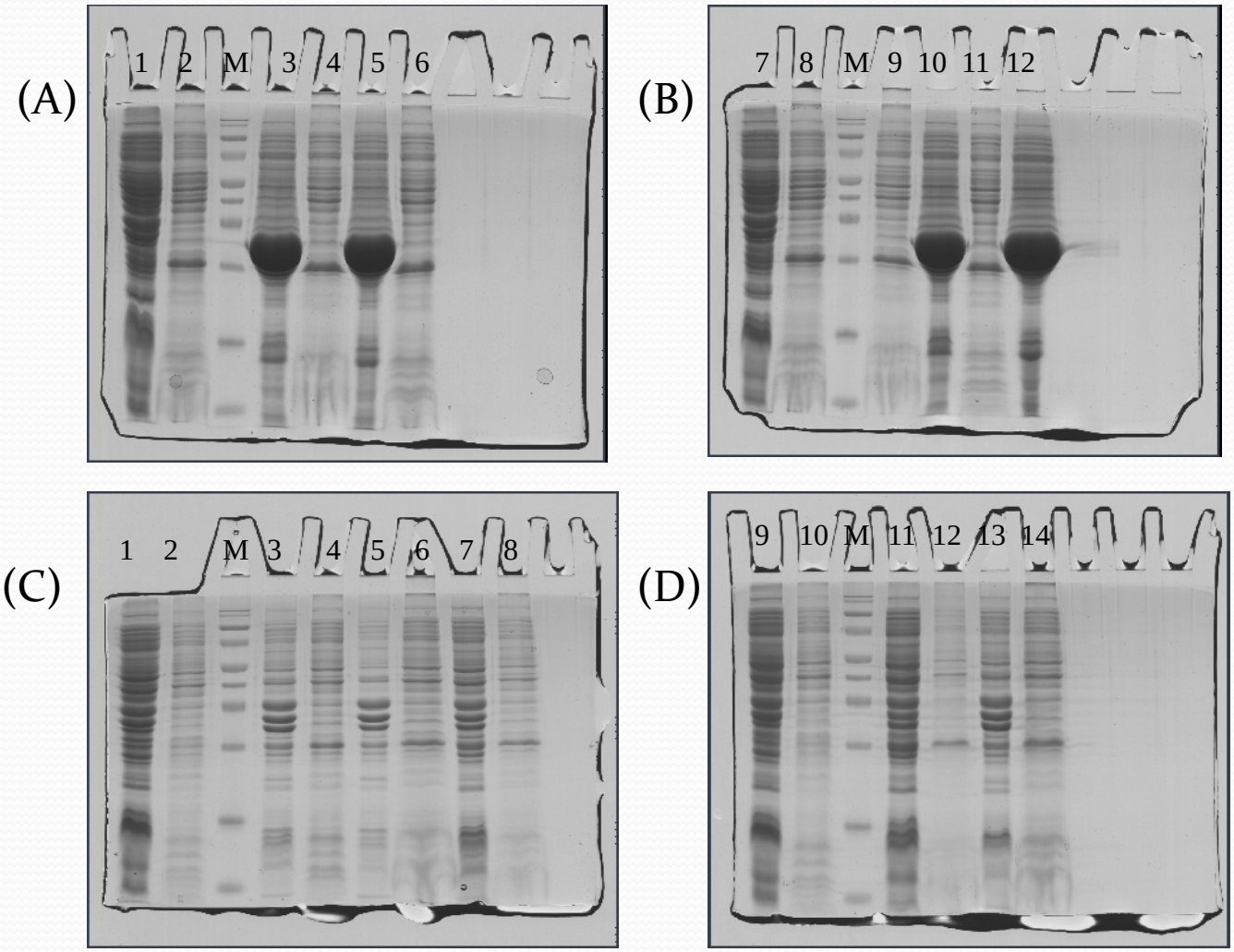
SDS polyacrylamide gel showing whole cell lysate of BL21 cells before and after induction with 0.5mM IPTG at 37^{o}C for 4 hours at 220rpm. Lane 1, 3 depicts uninduced condition. Lane 2, 4 depicts whole cell proteins after induction. The thick band in lanes 2 and 4 represents KPC-2 protein. Lane M shows protein markers.

Optimization of Conditions for Purification of Recombinant KPC-2 Protein in Soluble From

Conditions

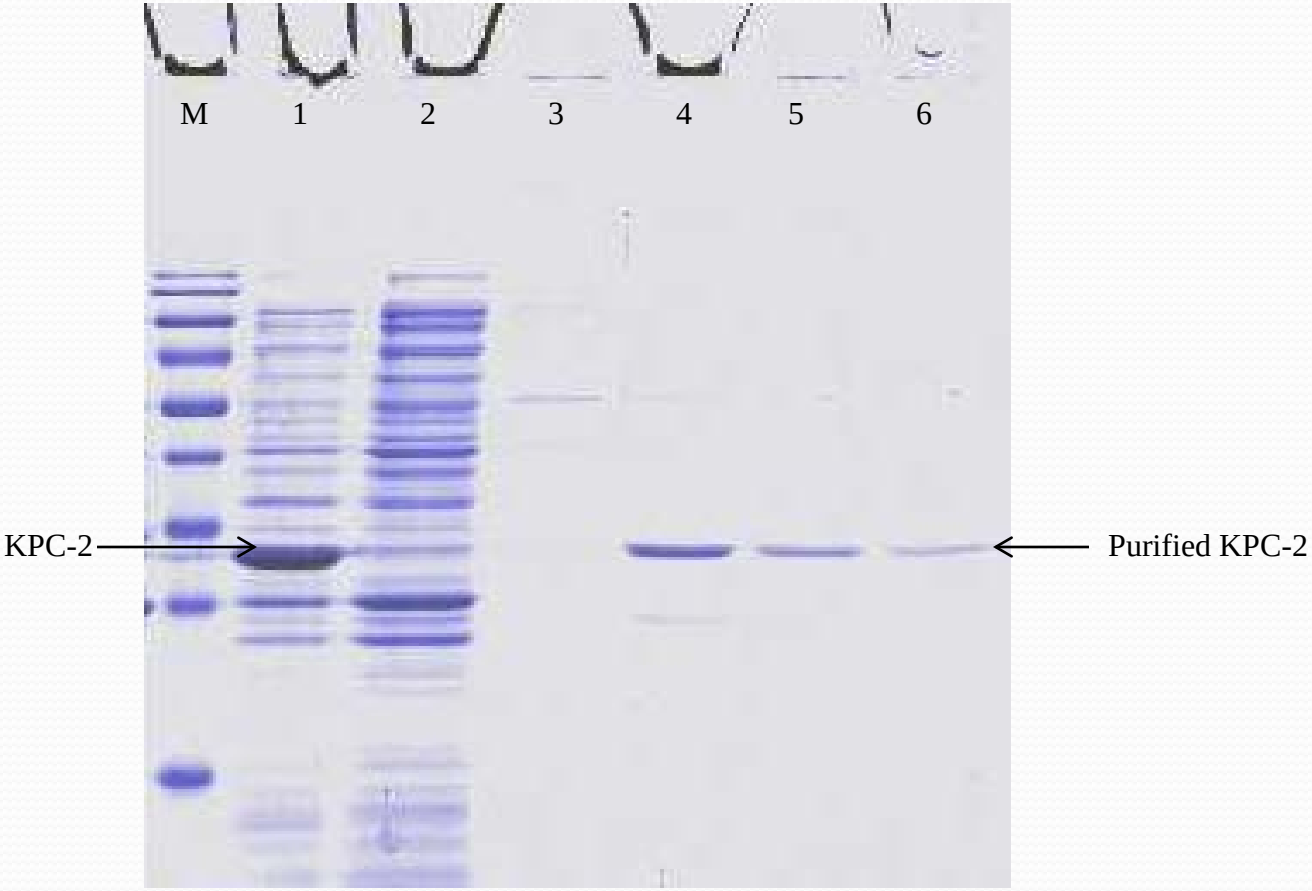
1. Induced with 0.1mM, 0.25mM, 0.5mM, 0.75mM and 1mM IPTG and grown at 37^0C, 220 rpm for 4 hours.
2. Induced with 0.1mM, 0.25mM, 0.5mM, 0.75mM and 1mM IPTG and grown at 25^0C, 220 rpm for 15 hours.
3. Induced with 0.1mM, 0.25mM, 0.5mM, 0.75mM and 1mM IPTG and grown at 20^0C, 220 rpm for 15 hours.

Whole cell lysates of *E. coli* BL21 cells grown at 37 ^{0}C and 25 ^{0}C



SDS Gel showing protein profile of bacterial cell pellet and supernatant after sonication.. (A) and (B) grown at 37^{0}C. (C) and (D) grown at 25^{0}C. IPTG concentration at 0.1mM, 0.25mM, 0.5mM, 0.75mM and 1mM .

Purification of Recombinant KPC-2 Protein



SDS PAGE showing purified His-tagged protein KPC-2 from bacterial lysate. Lane 4, 5, 6 shows purified protein in subsequent elutions. Lane 1 shows soluble protein in supernatant.

In view of the above background, we initiated our study on *Klebsiella pneumoniae* carbapenemase (KPC-2).

This Ambler class A enzyme is resistant against carbapenems, which are the last choice drugs for severe infections caused by multidrug resistant Gram negative bacteria.

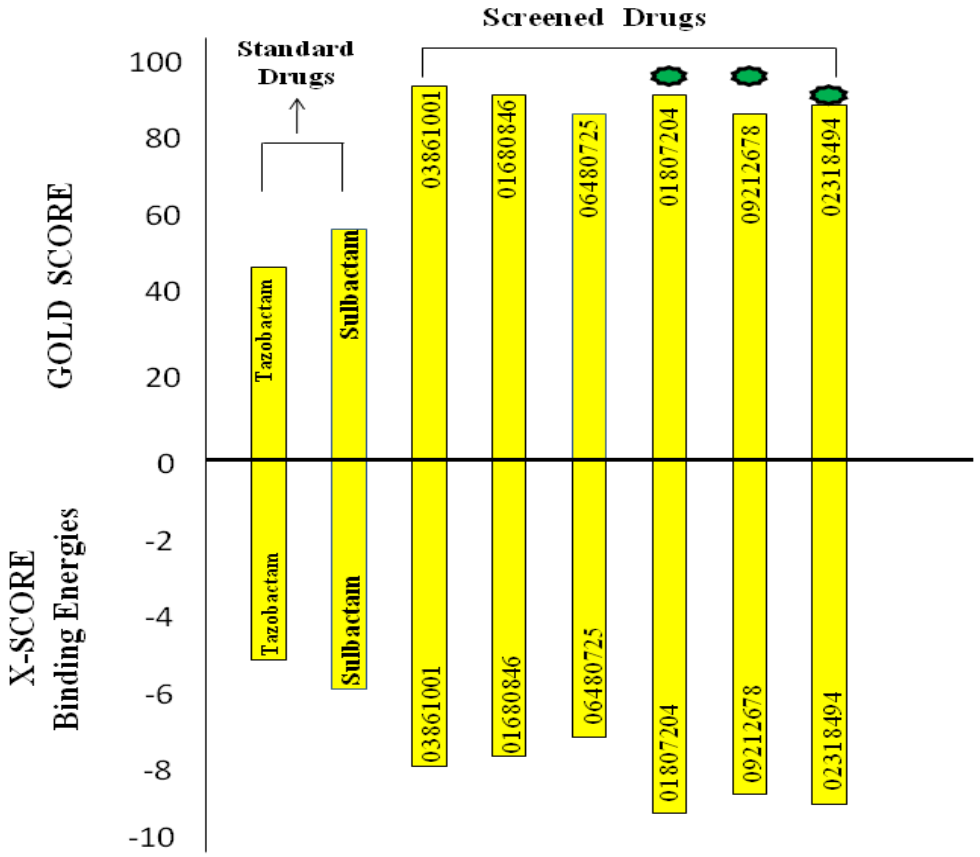
With increasing resistance in disease causing bacteria and very few new antibiotics in development, to maintain the efficiency of current antibiotics by combining them with efficient inhibitors is utterly important. But. . .

Recent studies indicated that KPC-2 is not inactivated by classical β-lactamase inhibitors.

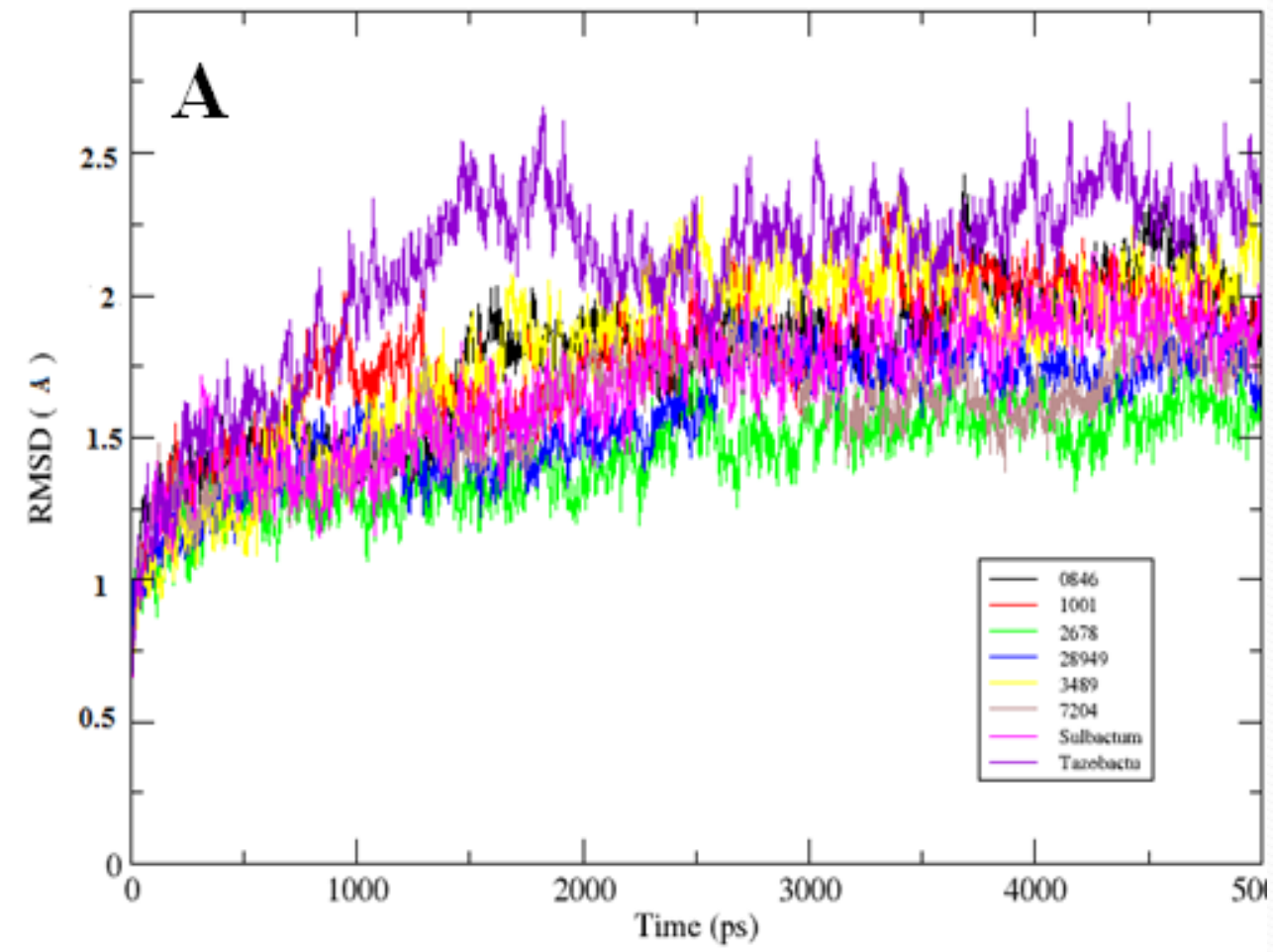
So we need to search for new and potent inhibitors against KPC-2 to keep the resistance menace in check.

GOLD Fitness score and binding energies of reference and screened compounds.

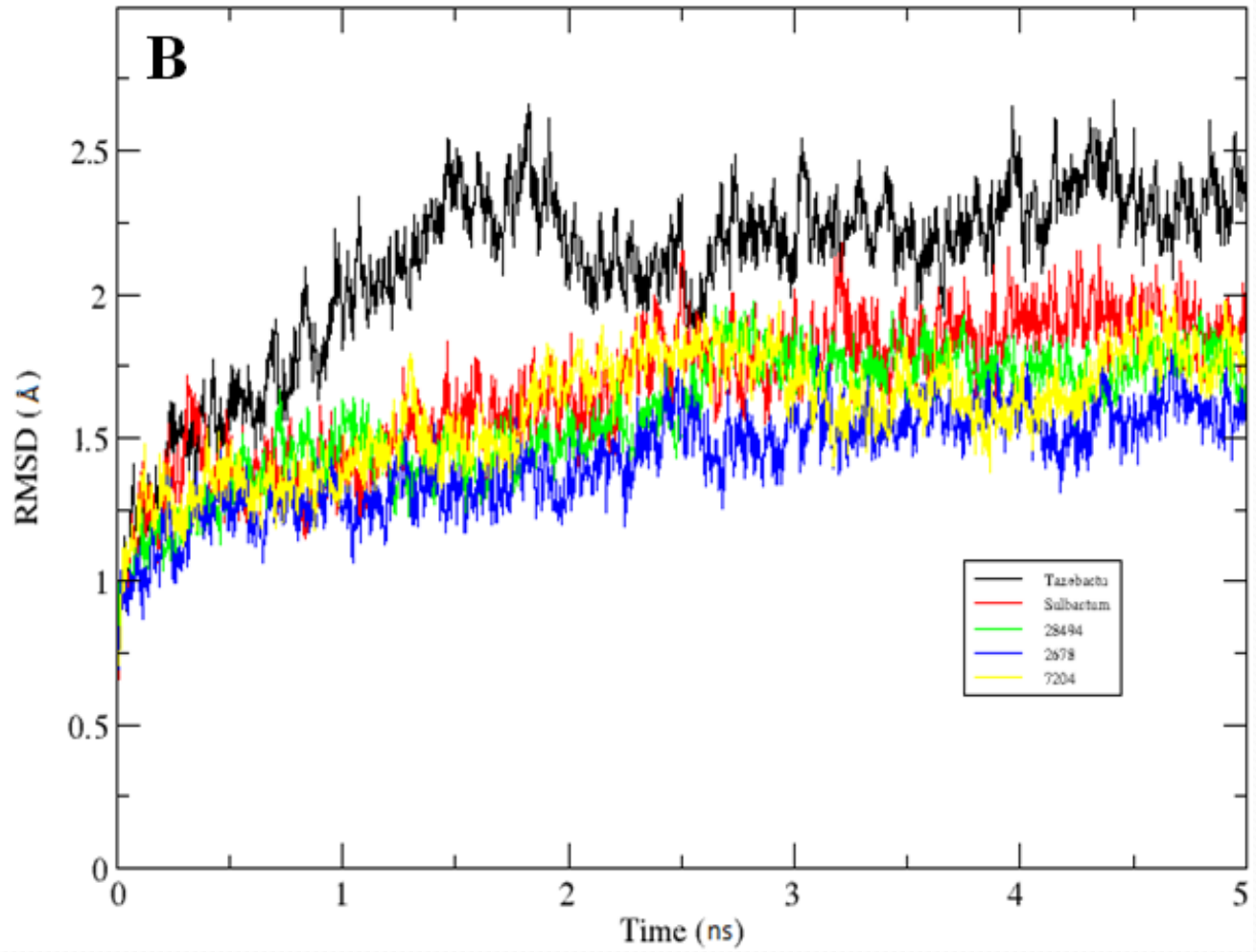
Compound ID	GOLD Fitness	X-Score Binding Energies (Kcal/mol)
ZINC03861001	91.93	-8.35
ZINC01680846	87.82	-6.99
ZINC02318494	82.80	-8.50
ZINC01807204	84.91	-8.90
ZINC06480725	80.37	-8.28
ZINC09212678	84.87	-7.52



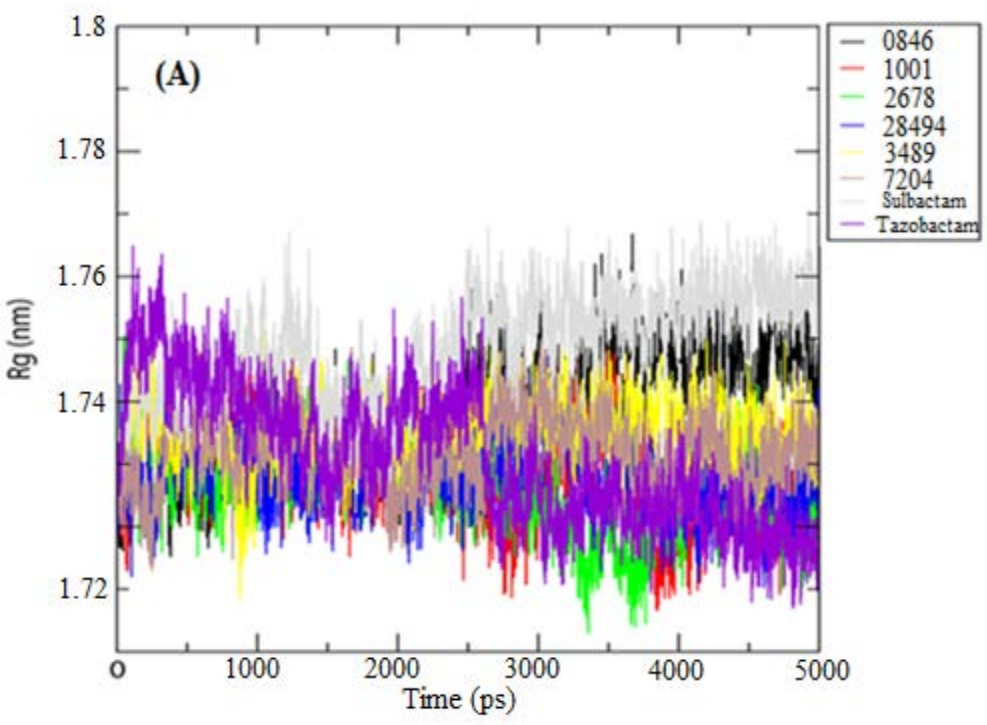
Plot of RMSD vs time of all the selected complexes



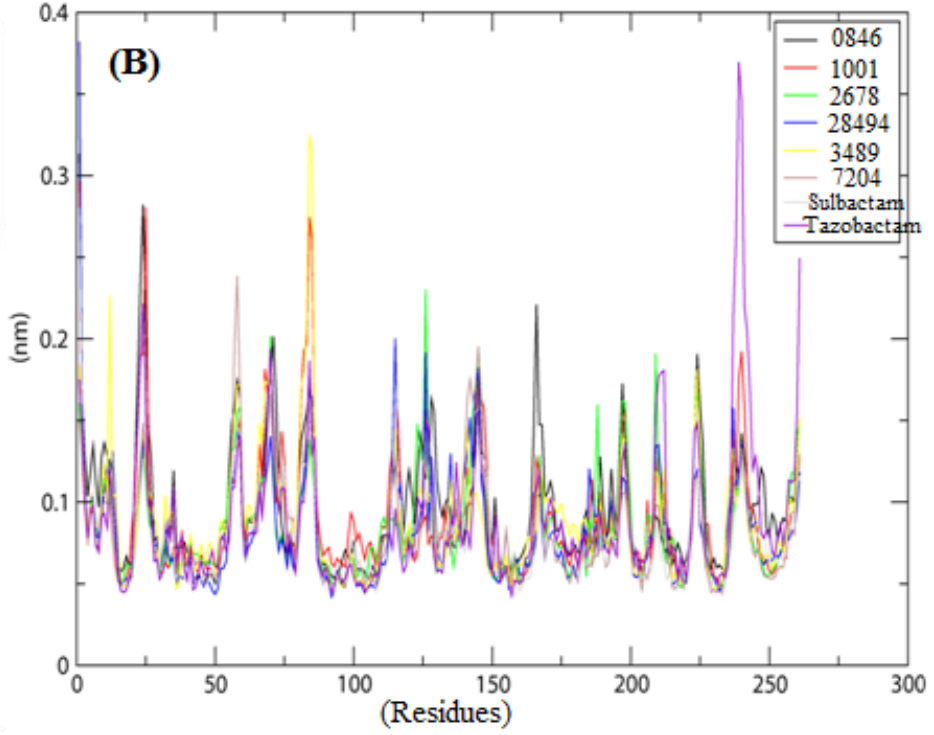
Plot of RMSD vs time of best three molecules



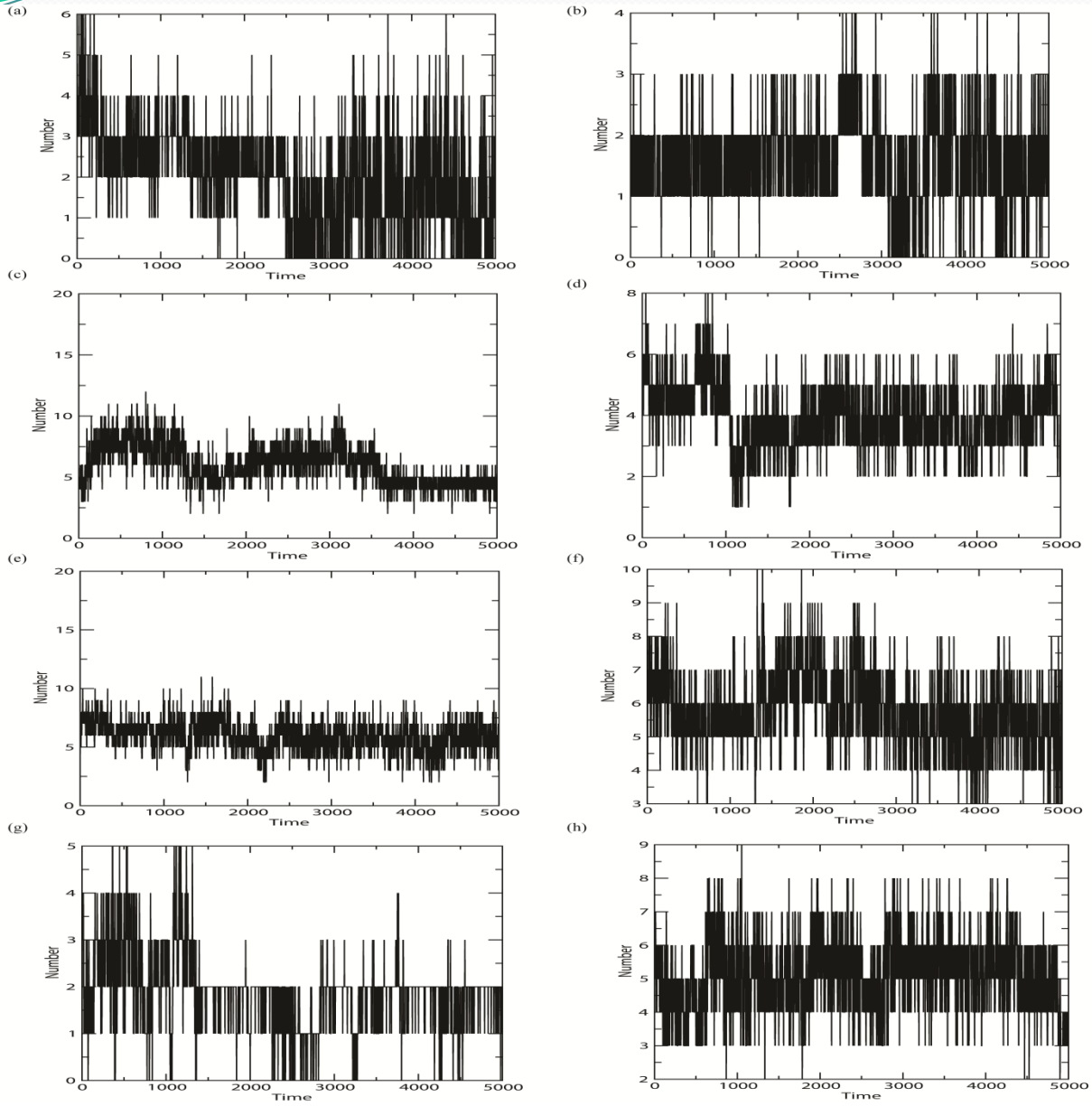
Radius of Gyration



Root Mean Square Flutuation

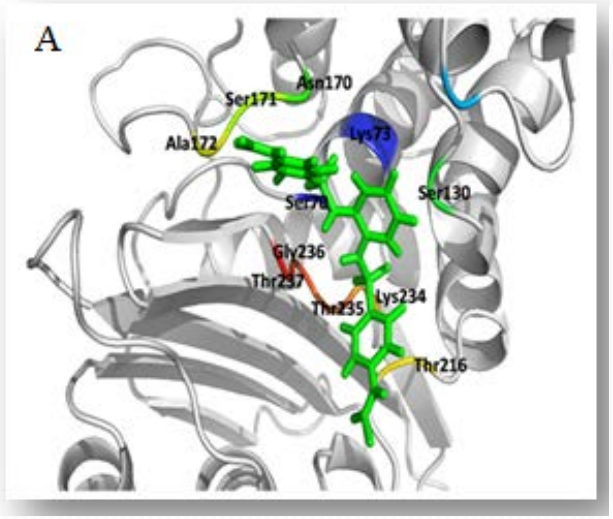


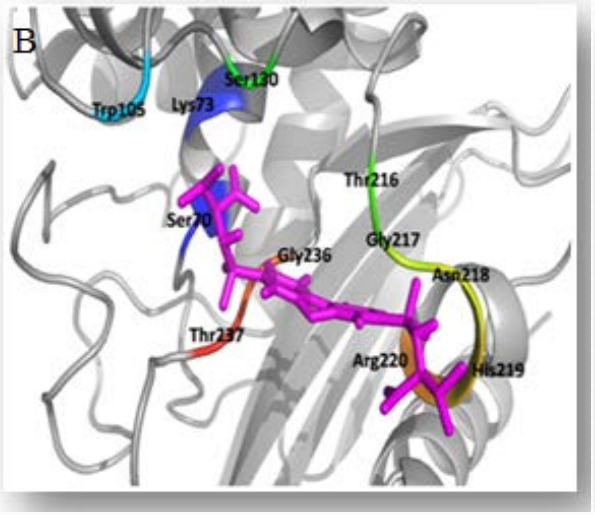
(A) Plot of Radiation of gyration(Rg) of all the selected complexes. (B) Cα root mean square fluctuation of the complexes obtained during 5ns MD simulations.

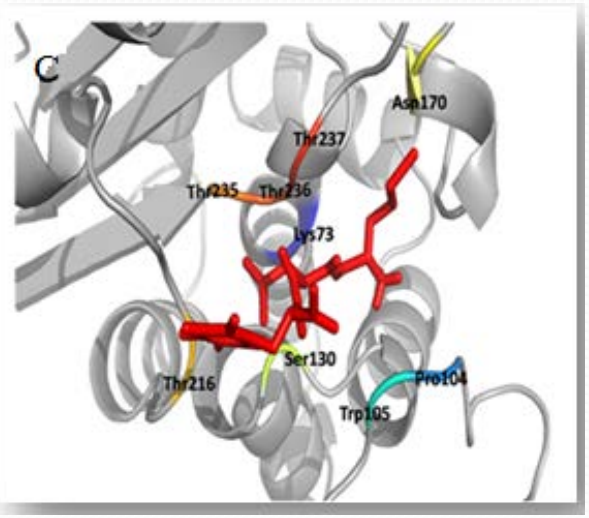


Evaluation of ligand-enzyme interaction by the number of hydrogen bonds at a function of time
(a) ZINC01680846,
(b) ZINC03861001
(c) ZINC09212678,
(d) ZINC06480725
(e) ZINC1807204,
(f) ZINC02318494,
(g) Sulbactam,
(h) Tazobactam.

Binding orientation of most active compounds

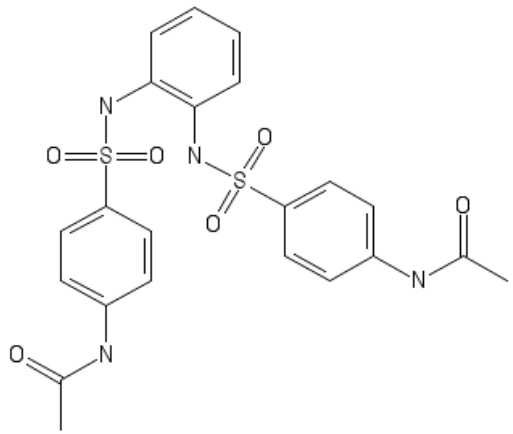




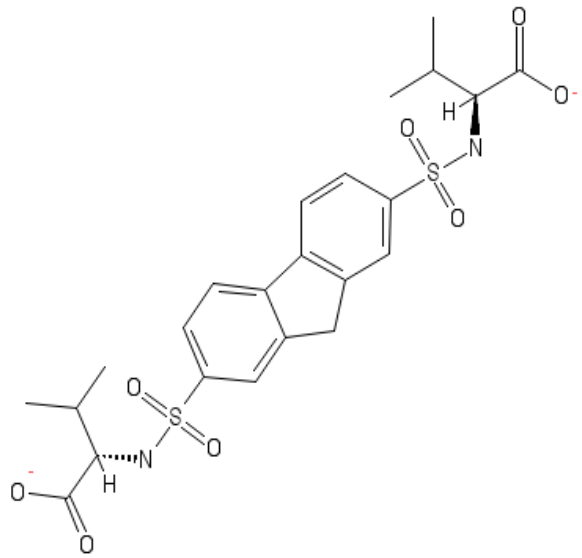


Compound	Residues involved in Hydrogen Bonding	Residues involved in Hydrophobic interaction	H-bond Range(A)	Hydrophobic interaction	No of Non-bond
Sulbactam	Arg220	Thr216, Thr235, Thr237,	2.97	2.50 - 3.80	0
Tazobactam	Ser70, Thr237	Arp105, Thr235	2.75 - 2.77	2.7 - 3.80	7
ZINC03861001	Ser70, Lys73, Trp105, Ser130, Thr237	Ser70, Trp105, Asn132, Asn170, Gly236, Cys238	2.64 - 2.90	2.97 - 3.90	34
ZINC01680846	Ser130, Asn170, Ala172, Thr235, Cys238	Thr235, Thr237, Cys238,	2.83 - 3.10	3.24 -3.90	22
ZINC02318494	Ser70, Lys73, Gly21, Asn218, His219, Thr237	Ser70, Trp105, Ser130, Thr216, Gly217, His219, arg220, Gly236,Thr237	2.75 - 2.99	3.48 -3.89	23
ZINC01807204	Ser70, Lys73, Ser130, Ala172, Lys234, Thr235, Thr237	Ser70, Trp105, Ser130, Asn10, Ser170, thr216, Thr237, Cys238	2.50 - 3.25	3.35 -3.80	34
ZINC06480725	Ser70, Ser130, Asn170	Trp105, Asn132, Glu166, Leu167	2.56 -3.1	3.16 - 3.89	29
ZINC09212678	Lys73, Pro104, Thr237	Trp105, Ser130, Asn170, Thr216, Thr235, Thr237	2.69 - 3.01	3.39 - 3.84	15

Chemical structure of screened inhibitors



ZINC01807204



ZINC02318494

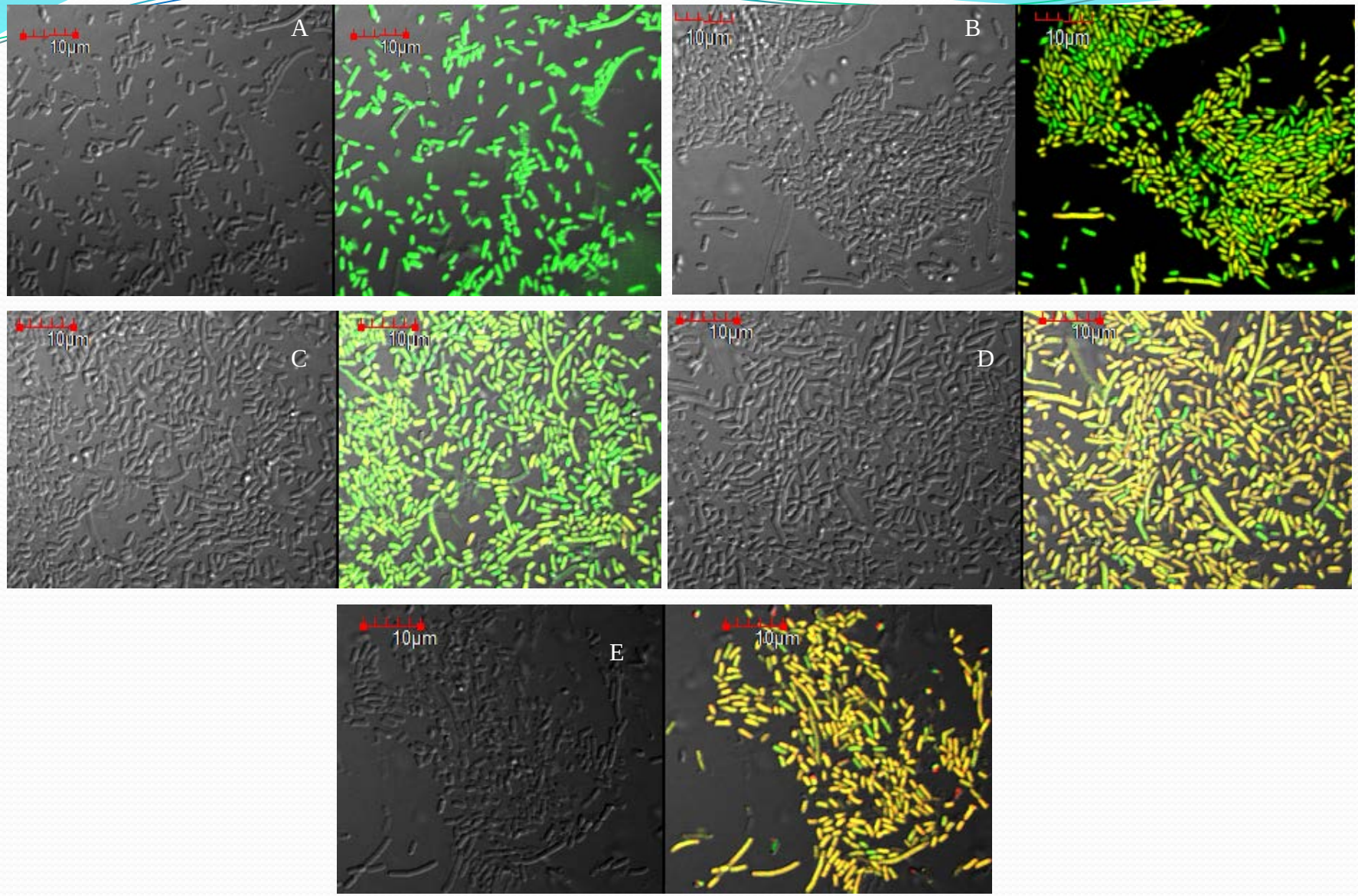
MICs of β-lactam antibiotics alone and in combination with inhibitors for *E. coli* BL21/DH5α transformed with recombinant bla_{KPC-2} from *Klebsiella pneumoniae*.

	NP6 (pQE2-KPC)DH5α	**(pQE2-Original)DH5α**	**DH5α**
Cefoxitin[a]	≥16	1	1
Cefoxitin+Tazobactam	16	1	1
Cefoxitin+Salbactum	1	1	1
Cefoxitin+01807204	**8**	**2**	**1**
Cefoxitin+02318494	**8**	**2**	**0.25**
Ceftazidime[b]	≥8	0.125	0.125
Ceftazidime+Tazobactam	8	0.125	0.25
Ceftazidime+Sa bactum	8	0.125	0.25
Ceftazidium+01807204	**2**	**0.125**	**0.25**
Ceftazidium+02318494	**2**	**0.125**	**0.2**
Ceftriaxone[a]	≥128	0.0625	0.0312
Ceftriaxone+Tazobactam	64	0.0625	0.0156
Ceftriaxone+Salbactum	64	0.0625	0.0156
Ceftriaxone+01807204	**64**	**0.125**	**0.0156**
Ceftriaxone+02318494	**64**	**0.125**	**0.0156**
Cefepime[a]	≥8	0.5	0.25
Cefepime+Tazobactam	8	0.5	0.0156
Cefepime+Salbactum	4	0.5	0.0156
Cefepime+01807204	**4**	**0** **5**	**0.0156**
Cefepime+02318494	**4**	**0.5**	**0.0156**

MIC *continued…*

Antimicrobial Agents	MIC (µg/ml)	
	BL21 (pQE2-KPC-2)	BL21 (Null plasmid)
Imipenem	≥64	0.25
Imipenem+tazobactam	32	0.25
Imipenem+sulbactam	64	0.25
Imipenem+clavulanic acid	32	0.125
Imipenem+ZINC01807204	16	0.125
Imipenem+ZINC02318494	16	0.25
Meropenem	≥64	0.125
Meropenem+tazobactam	16	0.0625
Meropenem+sulbactam	32	0.125
Meropenem+clavulanic acid	16	0.625
Meropenem+ZINC01807204	8	0.125
Meropenem+ZINC02318494	8	0.0625
Ertapenem	≥128	0.25
Ertapenem+tazobactam	32	0.25
Ertapenem+sulbactam	64	0.125
Ertapenem+clavulanic acid	32	0.25
Ertapenem+ZINC01807204	16	0.125
Ertapenem+ ZINC02318494	16	0.25

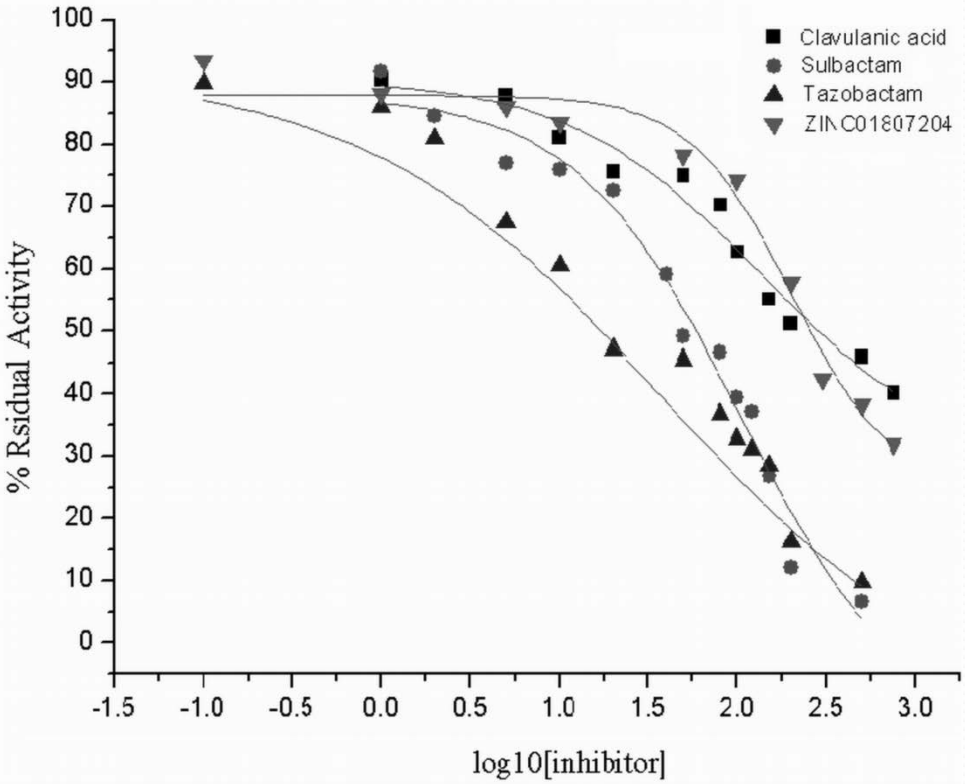
CLSM images of *E. coli* BL21 transformants harbouring bla_{KPC-2}



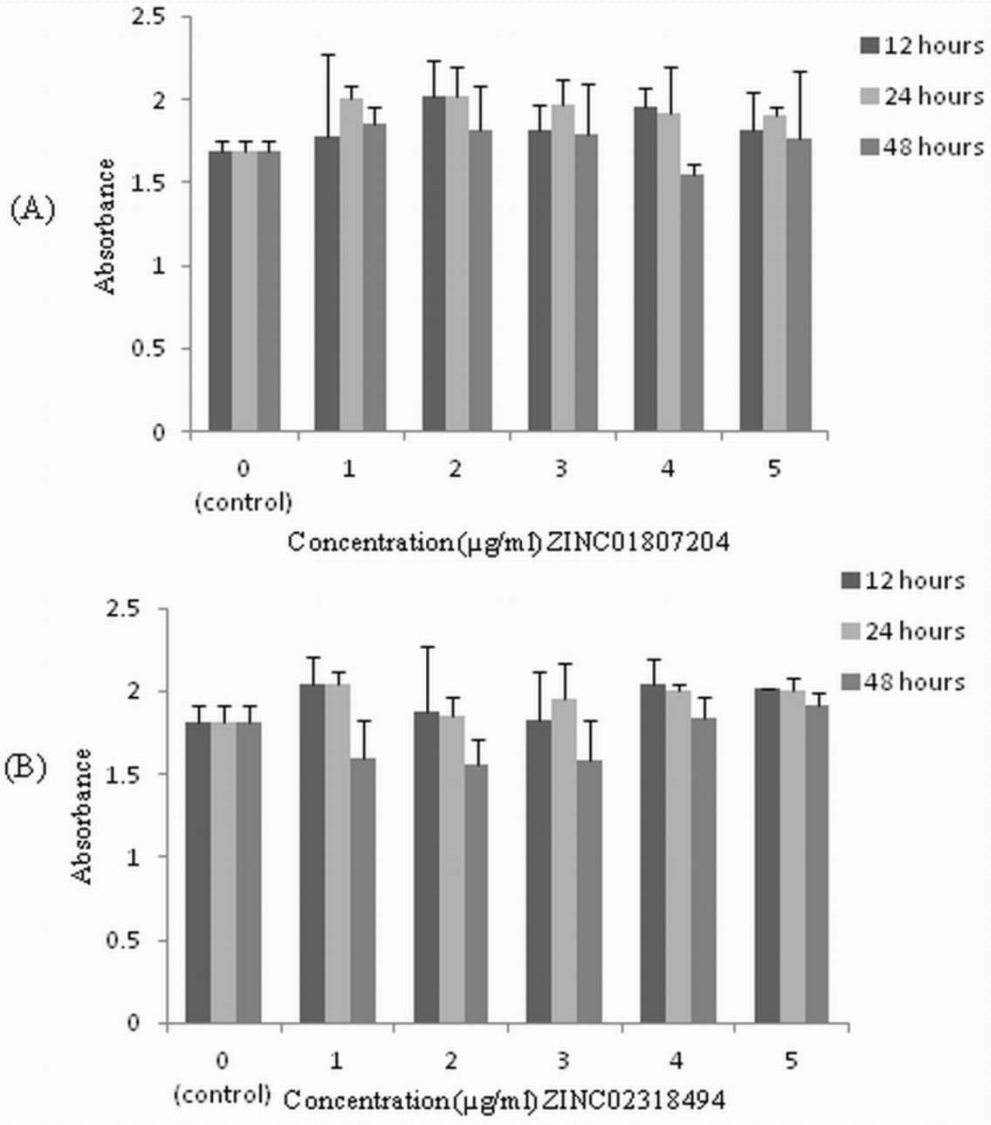
(A) Control, no treatment, B) Meropenem (C) Meropenem + tazobactam (D) Meropenem + ZINC01807204 (E) Meropenem + ZIC02318494

Half maximal inhibitory concentration values

Inhibitor	IC50 (µM)	Ki (µM)
Clavulanic acid	136.93	29.96
Sulbactam	106.09	23.21
Tazobactam	98.79	21.61
ZINC01807204	200.29	43.82
ZINC02318494	ND	ND



Toxicity determination by MTT Assay



Conclusion

- Our study concludes that ZINC01807204 is a novel non-β-lactam inhibitor.

- It competes for the active site of the KPC-2 and interacts non-covalently with key residues involved in β-lactam recognition and hydrolysis.

- The information gleaned from this study could be used to construct a wide variety of mechanisms-based inhibitors against KPC-2 producing bacteria.

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