African Journal of Microbiology Research Vol. 6(9), pp. 2010-2019, 09 March, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR11.1224
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Deeper in the antibiotics paradigm: Microbes react individually and collectively for spreading the resistance

Amro A. Amara[1,2*], Atef M. Shibl[2] and Abdul-Kader F. Tawfik[2]

[1]Protein Research Department, Genetic Engineering and Biotechnology Research Institute, Division of Microbiology, Mubarak City for Scientific Research and Technology Applications, Alexandria, Egypt.
[2]Division of Microbiology, Pharmaceutics Department, Faculty of Pharmacy at King Saud University (KSU) Riyadh, Kingdom of Saudi Arabia.

Accepted 27 December, 2011

There are exponential increases in the number of microbes acquired or which build resistance against various antibiotics. In this study we show that, a single strain, (*Escherichia coli* ATCC 8739 found in the protein database) has different *β*-lactamases. By investigating the Blast protein database for the existence of one of that different *E. coli β*-lactamases, the result shows that this protein can be found in hundreds of different microbes with 100% identity. Multiple alignment for both of *β*-lactamases of four *E. coli* strains alone or with sixteen other sequences represent *β*-lactamases of the classes A, B, C and D have been generated. A Phylogenic tree for the used *β*-lactamases shows that the four *E. coli β*-lactamases clustered with those of class C. Protein models for the four *E. coli β*-lactamases have been built from *β*-lactamases 3D structure using software modeller. Further more, the purpose of this study is to investigate other resistance mechanism. A model has been built to clarify the role of biofilm in the resistance elevation which can mimic what has been happened in reality. Alginate isolated from *Pseudomonas aeruginosa 9027* has been produced, purified and used. *E. coli* and *Bacillus stearothermophilus* ATCC 7953 were sensitive to Penicillin or streptomycin has been used to show the role of biofilm in resistance.

Key words: *β*-Lactamase, Biofilm, antibiotics resistance and protein model.

INTRODUCTION

Pathogenic microbes can be identified using molecular biology and or the biochemical tools (Robert and Pihet, 2008; Allerberger, 2003; Valenzuela, 2002). In parallel to that, various antibiotics sensitivity experiments usually used to determine the best antibiotic(s) for the treatment (Fox and Park, 2011; Chakraborti et al., 2010; Petrozzino et al., 2010; Deun et al., 2010; Domonoske and Severson 2009; Vrtis, 2008). However, the *in vitro* treatment could not guarantee the recovery due to the elevation of new resistance(s) (Daschner et al., 1987). The main reason is the global pollution with organo-chemical or the unwise treatments with the antibiotics (Nogales et al., 2011; Kümmerer, 2009). New strategies are in demand. Treating patients with broad spectrum antibiotics induce resistant (Lo and Cullen, 2006). The resistance to antibiotics happened mainly due to the acquiring of R-factor(s) or due to new mutation(s) in old but useless existing resistance gene(s) (Blizzard et al., 2010; Mendoza et al., 2009; De La Cueva-Méndez and Pimentel, 2007). Antibiotics resistance reduces the chance of the patient in recovery. Amara (2011); Amara and Hussain (2006); Hussain and Amara (2006) reported that those mutations could induce microbial variation under the strain level.

*Corresponding author. E-mail: amroamara@web.de. Tel: 4677730. Fax: 4676383.

TEM β-lactamase is the most prevalent one in Gram-negative enteric bacteria (Venkatachalam et al., 1994; Matagne et al., 1998). Venkatachalam et al. (1994) introduce amino acid substitutions in the active-site pocket of the β-lactamase. The experiments have been identified in natural isolates with increased resistance to extended-spectrum cephalosporins, such as cefotaxime and ceftazidime. Mutants were selected for 100-fold more ceftazidime resistance than wild-type. All mutants had a serine substitution at position 238, a lysine or arginine at position 240, and a small amino acid at position 241. The role of each substitution was investigated by constructing individual G238S, E240K, and R241G mutants as well as the G238, SE240K double mutant. The G238S mutant increases catalytic efficiency for both ceftazidime and cefotaxime. However, to achieve significant increases in catalytic efficiency, both G238S and the E240K mutants are required. The R241G mutant results in a small increase in catalytic efficiency for only ceftazidime. This example has been done in the lab; however nature is more dynamic and the probability that similar or diverse forms can be occur is very high. The existence of another protective mechanism in certain microbes can give the chance and the time for the resistance to be done, acquired and established. Spore forming bacteria can produce spore for protecting the microbes against antimicrobial agents till the condition become more suitable for germinating a vegetative cell (Fisher and Phillips, 2009; Helmann, 2006; Brett et al., 2005; Moyenuddin et al., 2002; Gröschel, 1996). Exopolysaccarid formation is another system for the protection (Amara et al., 2011).

Alginate can cause mechanical protection by coating or immobilizing the microbial cells. Hyperdization with resistant microbes can also exist naturally. Transformation can transfer R-factor harboring plasmid or integrate it in the genomic DNA (by transposon elements) and stable new gene or genes acquired (Clewell et al., 1995; Lupski, 1987; Tally and Malamy, 1986; Horaud et al., 1985; Tally et al., 1984). The studies done on the different microbes have neglected the role of the microbial community in the resistance elevation except in issues such as R-factor transformation. β-lactamase which is a subject for many studies which proved their wide diverse due to mutagenesis as a result of induced resistance has been a subject for investigation. This study is an investigation about β-lactamase resistance and the role of microbial strains individually and collectively in the resistance elevation. Both bioinformatics tools and experiments have been used to prove the role of single microbe or the microbial community in the elevation of resistance. Alginate has been used to mimic how sensitive strains can survive if it existed near the biofilm producing bacteria such as *Pseudomonas aeruginosa* (Alginate has been used to represent the *P. aeruginosa*). This survival can give the sensitive strains the time and the chance to gain or to build a real resistant structure.

The results obtained from this study proved that the mutation of certain β lactamase gene in a particular strain could happened individually (in the same strain) or collectively due to the interaction between different microbes. This paradigm of the microbial resistance become more complicated time after time and more sensitive investigation tools are in demand. The objective of this study is to analysis the β-lactames resistance diversity within one or more microbes on the level of protein structure/function. And to prove that one microbe could have different types of β-lactames while different microbes could have the same type due to mutation within the same species and transformation within different species. Biofilm as a mechanism involved in resistance has been also investigated.

MATERIALS AND METHODS

Microbial strain

The microbial strains used in this study to conduct experiments are *Escherichia coli* ATCC 8739, *Bacillus stearothermophilus* ATCC 7953 and *P. aeruginosa* ATCC 9027. *E. coli* ATCC 8739 has been used in the bioinformatic invastigations.
All bacterial strains were grown routinely in LB (Luria-Bertani) solid medium at 30°C (Sambrook, et al., 1989; Amara et al., 2011).

In flask alginate production at 37°c

The alginate has been produced from *P. aeruginosa* ATCC 9027 following the method described by Amara et al. (2011) and the static LB broth culture at 37°C has been used.

Alginate purification and characteriation

50 mM EDTA has been added to the culture and the mixture has been vortex gently. The cells have been separated from the supernatant using centrifugation at 1500 rpm for 10 min. The supernatant then collected and double volume of cold ethanol has been added. The mixture then has been left in -70°C refrigerator for 3 h (Amara et al., 2011). The alginate precipitate has been collected by centrifugation at 13000 rpm. The precipitate then dialyzed against distilled water (pH 8) in 2 L of water volume at room temperature and gentle stirring. The dialysis continued for 24 h. The alginate then collected and dried. The alginate has been examined using FT-IR spectrophotometer. One mg of pure dried alginate was used for IR analysis following the method described by Sherbrock-Cox et al. (1984) and Amara et al. (2011). The IR spectrum was compared with a known alginate sample using Shimadzu-Spectrophotometer.

Agar disc diffusion methods (antibiotics-sensitivity test)

Conventional agar disc diffusion method was performed, as described in NCCLS documents (NCCLS, 1999; Kiehlbauch et al., 2000), to determine the antibiotic sensitivity (Table 1). The used antibiotics as disks are IPM-10 (Primaxin TM-Cerbapenem), TE-15 (TelithromycinTM-Ketolide), SXT (Sulfamethoxazole/Trimethoprin-Folic acid pathway inhibitor), ATM-30 (Aztreonam-Monobactam), CRO-30 (RocephinTM-Cephem/Cephalosporin II), FOX-30 (Mefoxin-Cephem/Cephamycin/Cephalosporin II) and AMC-3

Table 1. Antibiotic sensitivity test.

Antibiotic names	Microbial names and antibiotic sensitivity results		
	***E. coli* ATCC 8739**	***B. stearothermophilus* ATTC 7953**	***P. aeruginosa* ATCC 9027**
IPM-10	s	s	s
TE-15	r	s	r
SXT	r	r	r
ATM-30	r	r	s
CRO-30	r	r	r
AMC-3	r	r	r
Pinicillin/Streptomycin 5 µl/disk	s	s	r

(Augmentin-*β*-Lactam/*β*–Lactamase inhibitor combination). Pinicillin/Streptomycin [Gibco BRL-Life technologyies Inc. USA (5000 units Pinicillin /ml: 5000 µg/ml Streptomycin sulphate upon dehydration with 20 ml water)] has been prepared manually and 5 µl added on a filter paper disk (For each one µl contain 5 units penicillin and 5 µg streptomycin). The different used antibiotic disks have been loaded on the surface of NA plates (Instead of Muller-Hinton agar) contain the tested organisms (on its surface). The tested organisms are: *E. coli* ATCC 8739, *B. stearothermophilus* ATTC 7953 and *P. aeruginosa* ATCC 9027. The plates with the tested strains and antibiotic disks have been incubated overnight at 37°C. The diameter of the zone of inhibition for each antibiotic has been determined and the results represented as sensitive (s), resistant (r) or moderate (m).

Study of the performance of the antibiotic in the presence of alginate

An experiment was designed same to the above antibiotic disk diffusion method. After spreading *E. coli* ATCC 8739 and *B. stearothermophilus* ATTC 7953 (sensitive to P/S and unable to produce alginate) on the surface of the plat. The plat left for the complete dryness and to allow the organism to establish themselves on its surface. Different concentration of the alginate has been used and represent concentrations equal to 0, 333, 666, 1000 and 1333 µg/ml. On the surface of each plat 50 µl of the alginate different concentration has been loaded each in one plat respectively just beside the center of the plat. The antibiotic disk then put in the center of the plat. Plates incubated at 37°C for overnight and the effect of alginate has been evaluated.

Multiple alignments, phylogenic tree and protein models

Multiple alignment for four *β*-lactamases protein sequances (collected from the Blast-Database) of the *E. coli* ATCC 8739 strain alone or with sixteen other microbial *β*-lactamases have been generated (Clustal X version 2.0, BioEdit v. 7.0.9.0. and MEGA 5 have been used) (Hall 1999; Tamura et al., 2011; Larkin et al., 2007; Amara et al., 2009). Phylogenic tree for the used *β*-lactamases has been generated too (hllp://tree.bio.ed.ac.uk/software/figtree). Four protein models for each of the *E. coli* ATCC 8739 *β*-lactamases have been performed using the software Modeller v 9.8 (Webb et al., 2008; Marti-Renom et al., 2000; Sali and Blundell, 1993; Fiser et al., 2000). The modles have built from the well identified *β*-lactamases structures pdb files 2 whg.pdb (Lassaux et al., 2011); 2 wrs.pdb (Lassaux et al., 2010); 2 wzx.pdb (Clizzard et al., 2010) and 3if6.pdb (Docquier et al., 2010).

RESULTS AND DISCUSSION

The spreading of the antibiotics resistance causes a tremendous health problems. On other hand, the elevation of a resistant to a certain antibiotic put the producers under pressure. Companies loss the profits gained from selling this antibiotic and they are in need to invest in a new one. This investment is highly expensive, costly and time consuming. Amara and Hussain (2006) highlighting that (*P. aeruginosa* was the main example) using single disinfectant could elevate mutation as well as causing a big variation in the microbial community (Amara and Hussain, 2006; Hussain and Amara, 2006). Perhaps the clearest example which supports our speculation is the story of the Penicillin. This natural wonderful safe antibiotic and due to misuse become nearly useless. Even new Penicillin derivatives gained new resistance. *E. coli* *β*-lactamase has been selected to start the investigation. By searching for the *E. coli* ATCC 8739 *β*-lactamse using its name only 28 sequences have been obtained. From those sequences, four were with clear reference to *β*-lactamases for further investigations [*β*-lactamase 1: gi|169756765|gb|ACA79464.1| Beta-lactamase *[E. coli* ATCC 8739]; *β*-lactamase 2: gi|169754216|gb|ACA76915.1| beta-lactamase [*E. coli* ATCC 8739]; *β*-lactamase 3: gi|169756179|gb|ACA78878.1| beta-lactamase [*E. coli* ATCC 8739]; *β*-lactamase 4: gi|170021837|ref|YP_001726791.1| beta-lactamase [*E. coli* ATCC 8739]]. The multiple alignments for these four *β*-lactamase protein sequences and unexpectedly show a great variation between three of them while two are 100% identical as in Figure 1. Even so, 20 conserved amino acids only have been obtained. By applying one sequence (gi|169756765|gb|ACA79464.1| *β*-lactamase [*E. coli* ATCC 8739]) in the blast protein database hundred of sequences represent different microbial *β*-lactamases have been found with 100% identity. This proves that one' gene is different in the same strain (isolated from different sources) but same in hundred different microbes! *β*-Lactamase is a subject for modification in its main host (*E. coli* ATCC 8739 might not be its mother host) and it is a subject for transformation

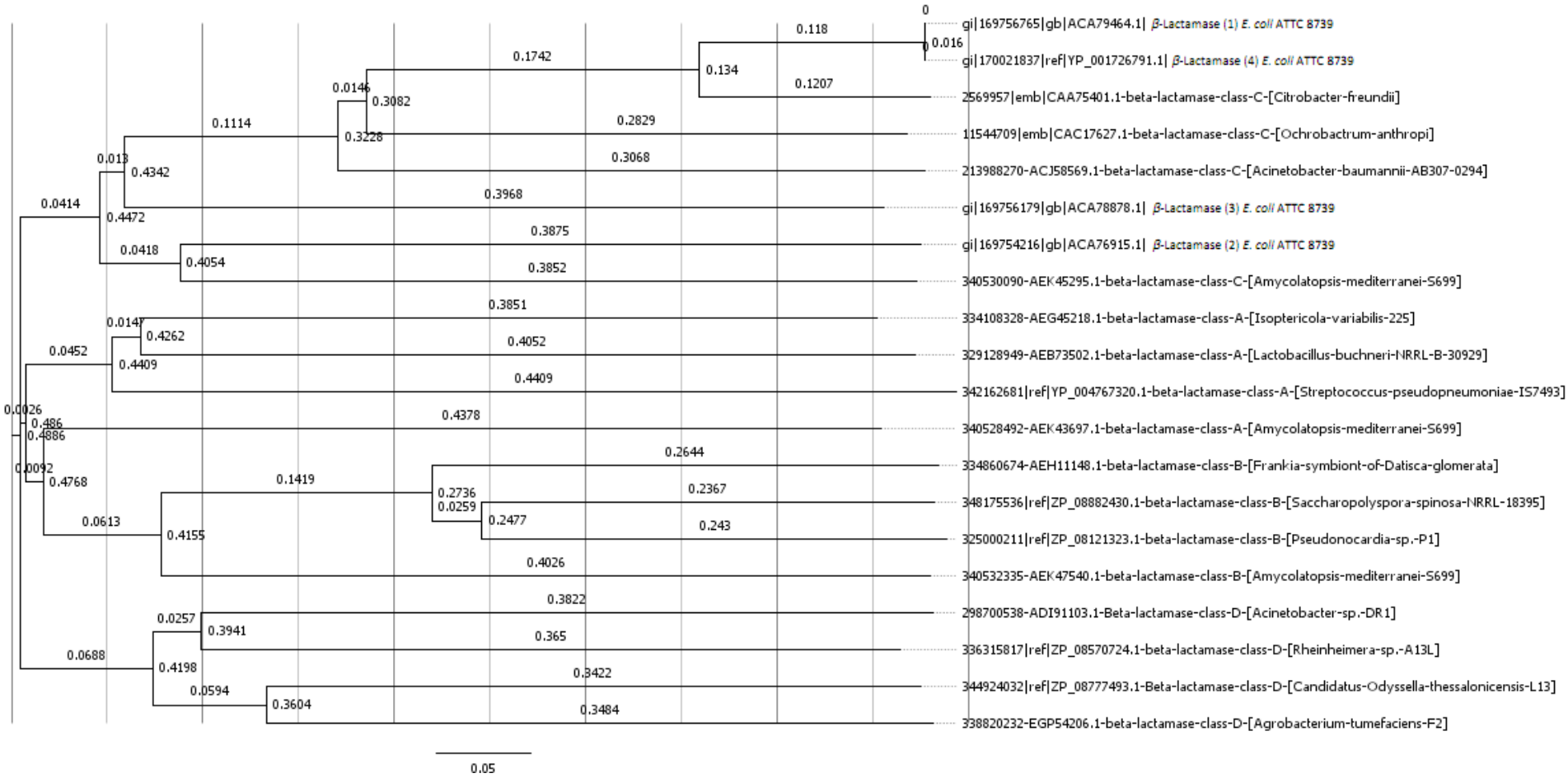
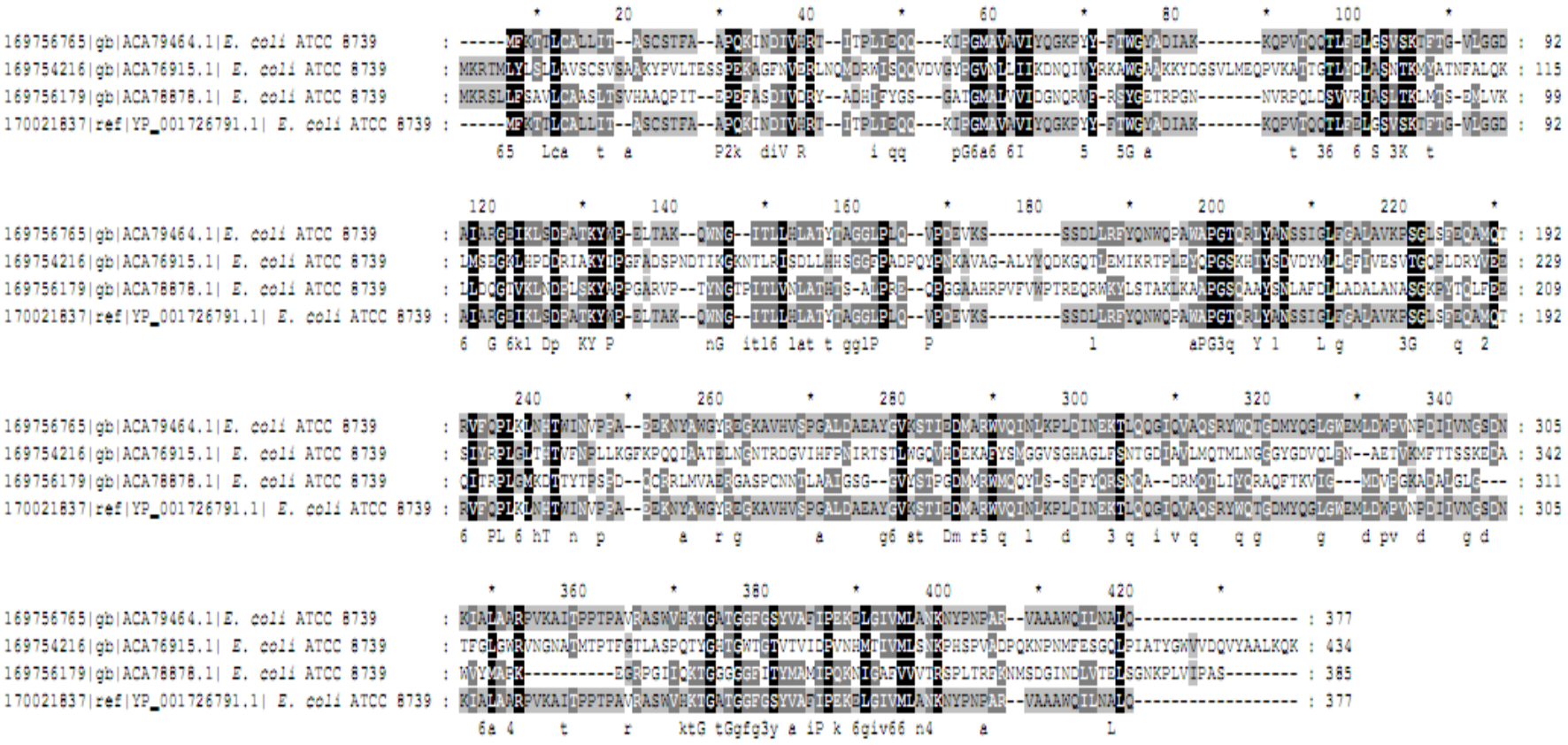


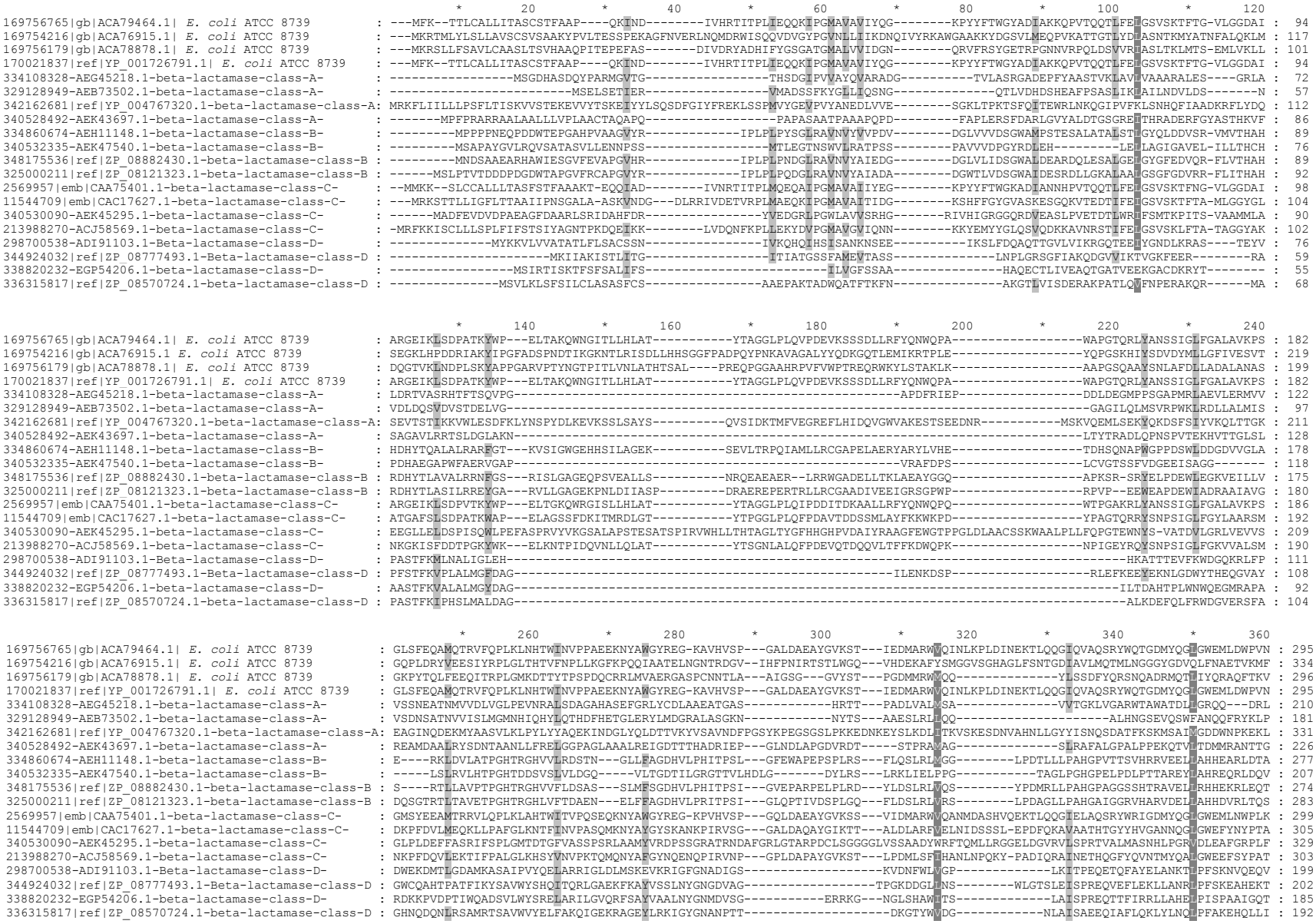
Figure 1. Phylogenetic tree of 20 *β*-lactamases. Four *β*-lactamases for *E. coli* ATCC 8739, Class A, B, C and D. The branching order and distance score were calculated by the program tree as described by Feng and Doolittle (1987).

within the different microbes. Whether *E. coli* ATCC 8739 might obtained another copy of *β*-lactamase from another microbe or not, we investigate the homology of the four used *β*-lactamases side by side with sixteen other *β*-lactamases. Each four *β*-lactamases represent class A, B, C or D. The multiple alignments and the phylogenic tree proved that the four *E. coli β*-lactamases have been clustered in the same class (C) (Figure 3) which is a strong indication that they are modified inside *E. coli* ATCC 8739 and not as a result of transformation. The multiple alignments in Figures 2 and 3 have proved that there is a big variation between the various *β*-lactamases. Even there are differences between three *β*-lactamases of the *E. coli* ATCC 8739 (one has used as control) but we have found 20 conserved amino acids [100%] (Figure 1). In



Figuer 2. Multiple alignment of primary structures of 4 *β*-Lactamases (*E. coli* ATCC 8739). Amino acids are shown in one-letter abbreviations. The faint shaded line show conserved amino acids while dark shaded represent 100% conserved amino acids.

contrast, when there is comparison in the level of classes (A, B, C and D), we obtained 0 conserved amino acids [100%] (Figure 3). Protein model for each of the different *E. coli* ATCC 8739 *β*-lactamases have been generated. The models proved that there is a critical change in the 3D structure of *β*-lactamases. The identical *β*-lactamases (Figure 4 (1, 4)) gave the same structure as a proof of the efficiency of the modeling. In fact the results obtained above is coming as a derivatives from our main plan in this study to investigate the role of alginate in protecting *β*-lactamases microbe to survive in nature against other combination (P/S) as a part of the collective microbial survival mechanism.



Figuer 3. Multiple alignment of primary structures of 4 *β*-Lactamases (*E. coli* ATCC 8739), 4 *β*-Lactamases of each of class A, B, C and D. Amino acids are shown in one-letter abbreviations. The shaded line show conserved amino acids.

Figure 3. Cont.

Microbes in our lab. represent *E. coli* ATCC 8739, *B. stearothermophilus* ATTC 7953 and *P. aeruginosa* ATCC 9027 have been investigated for the existence of *β*-lactamases. As we expected the three invastigated strains are resistance to *β*-lactam/*β*-lactam inhibitor. *P. aeruginosa* has been used to produce in lab. alginate which has been purified and characterized following the method described previously by Amara et al. (2011). The P/S combinations has been used to give a more contrast while *E. coli* ATCC 8739 as well as *B. stearothermophilus* are resistance to penicillin but sensitive to P/S. Alginate proves clearly that it has a supportive effect for the antibiotic to kill microbes in low concentrations while it has a protective effect in high concentrations as shown in Figure 5. The results show clearly that alginate could protect microbes from various antibiotics at the high concentration. This agree with the fact that alginate in low concentration is permeable and induces static condition for microbes. The survival due to high alginate concentration can either save the microbes or can give them the chance to elevate mutations or aquire resistance gene(s). This fact needs more studies to be fully understood. It is important to highlight that many authors have been proved that different mutation in *β*-lactamases conducted in lab released resistance to different antibiotics as the example of Venkatachalam et al. (1994) in the introduction part. The concept of one of the site directed mutagenesis kits (Promega) is based onthis fact. Another example explaining the dynamism of the genes transfer mechanisms is the entero-hemorrhagic *E. coli* isolates containing virulence plasmids and pathogenicity islands similar to those found in *Shigella spp*. (Burland et al., 1998 and Makino et al., 1998). Although it was a matter of speculation whether, those genetic deter-minants acquired, demonstrated that it contains as many as 1387 new genes in comparison with the previously sequenced nonpathogenic laboratory strain *E. coli* K-12 (Blattner et al., 1997). This study proves that the biological system is dynamic, and not only a single theory controlled the resistance generation and distr-ibution. Resistance could happened individually and collectively. Biofilm, which gave the chance and the time for sensitive strains to survive under the effect of different kind of antibiotics, it could be one of the essential mechanisms in antibiotic (such as P/S) resistance elevation.

ACKNOWLEDGMENT

The authors extend their appreciation to the Deanship of Scientific Research at king Saud University, for funding the work through the research group project no. RGP-VPP-038.

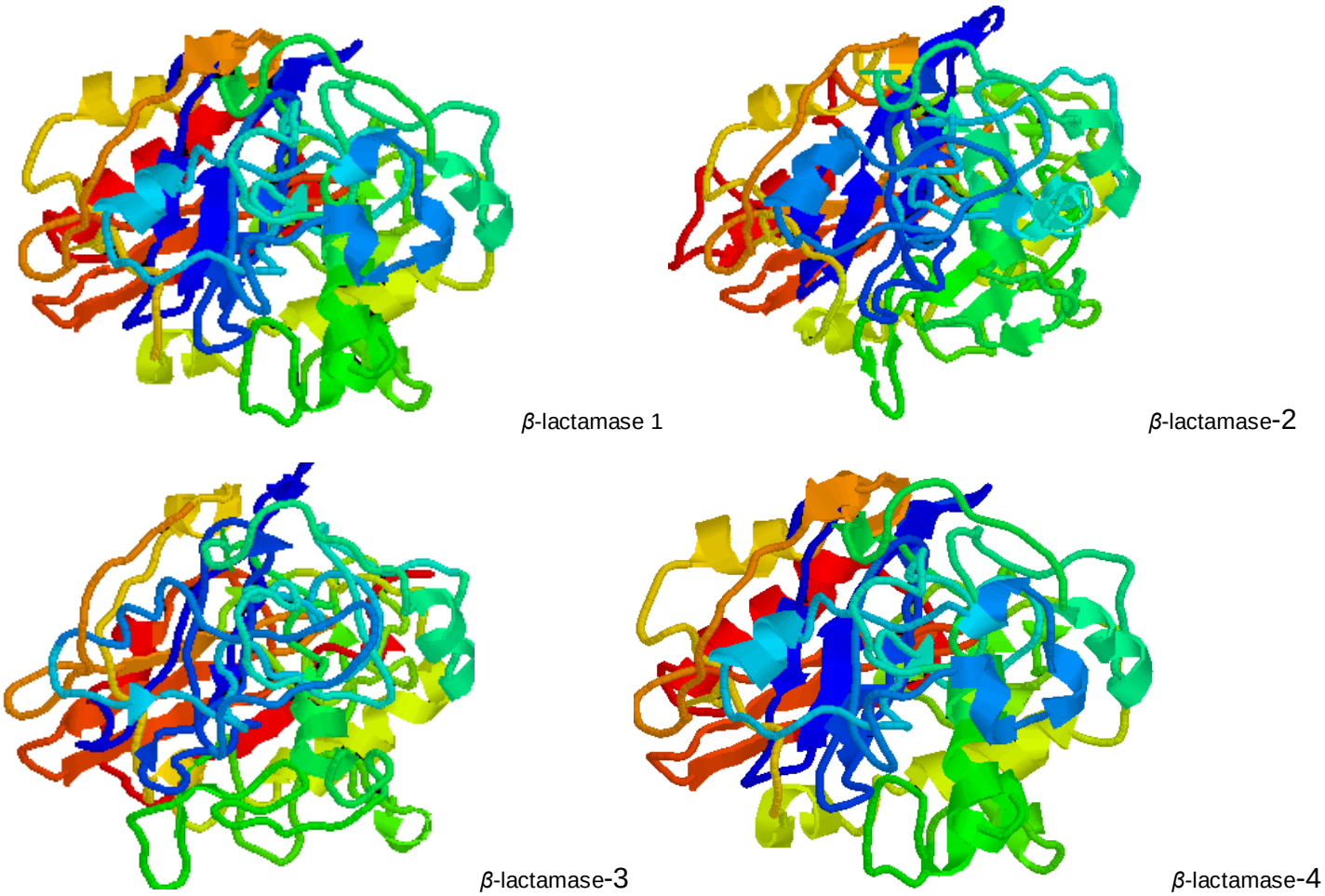


Figure 4. Threading model of the *E. coli* ATCC 8739 different β-lactamase [1 and 4 100% identical] 2D ulestereted by RasWin Molecular Graphics v 2.6.

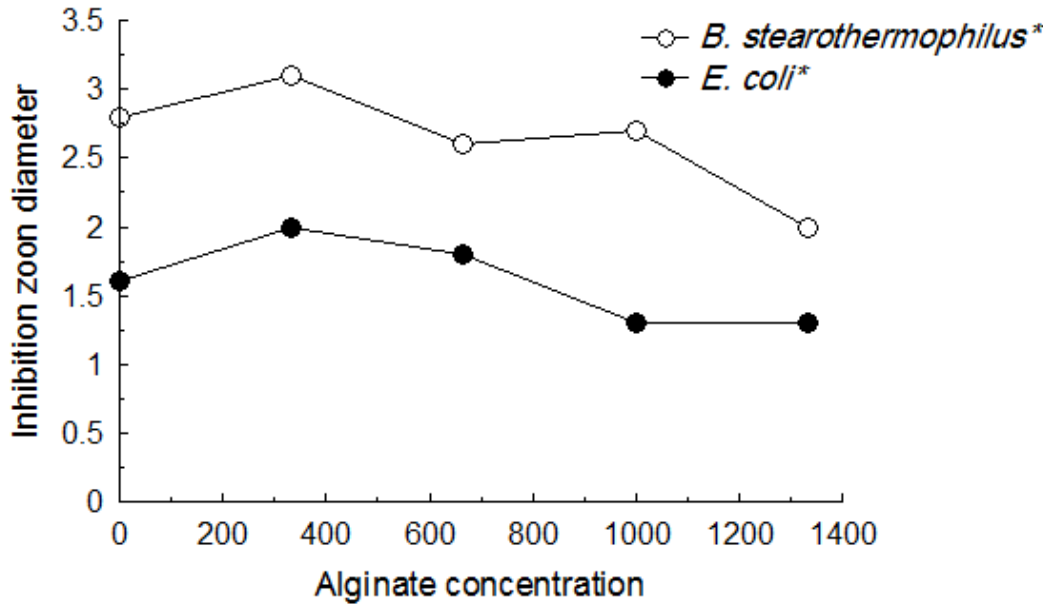


Figure 5. The inhibition zone diameter of Pinicilline/streptomycine

REFERENCES

Allerberger F (2003). Listeria: growth, phenotypic differentiation and molecular microbiology. FEMS. Immunol. Med. Microbiol., 35(3): 183-189.

Amara AA, Hassan MA, Abulhamd AT, Haroun BM (2011). *algs* genes: H_2O_2 Map their Viability and Validity in *Pseudomonas aeruginosa*. IJBB., pp. 321-330.

Amara AA, Hussain MZ (2006). Offline controlling of *Pseudomonas aeruginosa* resistant to protein inhibitor antibiotics using combination of EDTA and Na-citrate or disinfectant(s). Microbiology (China)., 33:95-100

Amara AA, Matar MS, Serour E (2009). Comparative study on the amino acids constituents of PhaC synthases Class I, II, III and IV EMBO – Conference- "Comparative genomics of Eukaryotic microorganisms" 2009, pp. 59.

Blattner FR, Plunkett G, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA, Goeden MA, Rose DJ, Mau B, Shao Y (1997). The complete genome sequence of *Escherichia coli* K-12. Science,
277: 1453–1474.

Blizzard TA, Chen H, Kim S, Wu J, Young K, Park YW, Ogawa A, Raghoobar S, Painter RE, Hairston N, Lee SH, Misura A, Felcetto T, Fitzgerald P, Sharma N, Lu J, Ha S, Hickey E, Hermes J, Hammond ML (2010). Side chain SAR of bicyclic beta-lactamase inhibitors (BLIs). 1. Discovery of a class C BLI for combination with imipinem. Bioorg. Med. Chem. Lett., 1:20(3): 918-921.

Brett MM, Hood J, Brazier JS, Duerden BI, Hahné SJ (2005). Soft tissue infections caused by spore-forming bacteria in injecting drug users in the United Kingdom. Epidemiol. Infect., 133(4): 575-582.

Chakraborti C, Le C, Yanofsky A (2010). Sensitivity of superficial cultures in lower extremity wounds. J. Hosp. Med., 5(7): 415-420.

Clewell DB, Jaworski DD, Flannagan SE, Zitzow LA, Su YA (1995). The conjugative transposon Tn916 of Enterococcus faecalis: structural analysis and some key factors involved in movement. Dev. Biol. Stand., 85: 11-17.

Webb DB, Shen MY, Sali A (2008). Protein structure modeling with MODELLER. Methods Mol. Biol., 426: 145-59.

Daschner F, Frank U, Just HM (1987). Proven and unproven methods in hospital infection control in intensive care units. Chemioterapia, 6(3): 184-189.

De La Cueva-Méndez G, Pimentel B (2007). Gene and cell survival: lessons from prokaryotic plasmid R1. EMBO. Rep., 8(5): 458-464.

Docquier J-D, Benvenuti M, Calderone V, Giuliani F, Kapetis D, Luca FDe, Rossolini GM, and Mangani S (2010). Crystal Structure of the Narrow-Spectrum OXA-46 Class D β-Lactamase: Relationship between Active-Site Lysine Carbamylation and Inhibition by Polycarboxylates Antimicrobial Antimicrob. Agents CH., 54(5): 2167-2174.

Domonoske C, Severson K (2009). Antimicrobial use and bacterial resistance in neonatal patients. Crit. Care Nurs Clin. North Am., 21(1): 87-95.

Feng DF, Doolittle RF (1987). Progressive sequence alignment as a prerequisite to correct phylogenetic trees. J. Mol. Evol., 25: 351-360.

Fiser A, Do RK, Sali A (2000). Modeling of loops in protein structures. Protein Sci., 9: 1753-1773.

Fisher K, Phillips C (2009). The ecology, epidemiology and virulence of *Enterococcus*. Microbiology, 155(Pt 6): 1749-1757.

Fox S, Park MA (2011). Penicillin skin testing in the evaluation and management of penicillin allergy. Ann. Allergy Asthma Immunol., 106(1): 1-7.

Gröschel DH (1996). *Clostridium difficile* infection. Crit. Rev. Clin. Lab. Sci., 33(3): 203-245.

Hall TA (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp., Ser., 41: 95-98.

Helmann JD (2006). Deciphering a complex genetic regulatory network: the *Bacillus subtilis* sigmaW protein and intrinsic resistance to antimicrobial compounds. Sci. Prog., 89(Pt 3-4): 243-2 66.

Horaud T, Le Bouguenec C, Pepper K (1985). Molecular genetics of resistance to macrolides, lincosamides and streptogramin B (MLS) in streptococci. J. Antimicrob. Chemother., 16 Suppl A: 111-135.

Hussain MZ, Amara AA (2006). Case-by-case study using antibiotic-EDTA combination to control *Pseudomonas aeruginosa*. Pak. J. Pharm. Sci., 19(3): 236-243

Kiehlbauch JA, Hannett GE, Salfinger M, Archinal W, Monserrat C, Carlyn C (2000). Use of the National Committee for Clinical Laboratory Standards guidelines for disk diffusion susceptibility testing in New York state laboratories. J. Clin. Microbiol., 38(9): 3341-3348.

Kümmerer K (2009). Antibiotics in the aquatic environment--a review--part II. Chemosphere, 75(4): 435-441.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007). Clustal W and Clustal X version 2.0. Bioinformatics, 23(21): 2947-2948.

Lassaux P, Hamel M, Gulea M, Delbrück H, Mercuri PS, Horsfall L, Dehareng D, Kupper M, Frère JM, Hoffmann K, Galleni M, Bebrone C (2010). Mercaptophosphonate compounds as broad-spectrum inhibitors of the metallo-beta-lactamases. J. Med. Chem., 53(13): 4862-4876.

Lo N, Cullen M (2006). Antibiotic prophylaxis in chemotherapy-induced neutropenia: time to reconsider. Hematol Oncol., 24(3): 120-125.

Lupski JR (1987). Molecular mechanisms for transposition of drug-resistance genes and other movable genetic elements. Rev. Infect. Dis., 9(2): 357-368.

Makino K, Ishii K, Yasunaga T, Hattori M, Yokoyama K, Yutsudo CH, Kubota Y, Yamaichi Y, Iida T, Yamamoto K, Honda T, Han CG, Ohtsubo E, Kasamatsu M, Hayashi T, Kuhara S, Shinagawa H (1998). Complete nucleotide sequences of 93-kb and 3.3-kb plasmids of an enterohemorrhagic *Escherichia coli* O157:H7 derived from Sakai outbreak. DNA Res., 5: 1–9

Marti-Renom MA, Stuart A, Fiser A, Sánchez R, Melo F, Sali A (2000). Comparative protein structure modeling of genes and genomes. Annu. Rev. Biophys. Biomol. Struct., 29: 291-325.

Matagne A, Lamotte-Brasseur J, Freare J-M (1998). Catalytic properties of class A b-lactamases: efficiency and diversity. J. Biochem., 330: 581-598.

Mendoza Mdel C, Herrero A, Rodicio MR (2009). [Evolutionary engineering in Salmonella: mergence of hybrid virulence-resistance plasmids in non-typhoid serotypes]. Enferm. Infect. Microbiol. Clin., 7(1): 37-43.

Moyenuddin M, Williamson JC, Ohl CA (2002). *Clostridium difficile*-associated diarrhea: current strategies for diagnosis and therapy. Curr. Gastroenterol, Rep., 4(4): 279-286.

National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial susceptibility testing. Ninth informational supplement. Wayne, Pennsyslvania: NCCLS (1999). document M100-S9; 19: 1, Table 21.

Nogales B, Lanfranconi MP, Piña-Villalonga JM, Bosch R (2011). Anthropogenic perturbations in marine microbial communities. FEMS Microbiol. Rev., 35(2): 275-298.

Lassaux P, Traoré DAK, Loisel E, Favier A, Docquier J-D, Sohier JS, Laurent C, Bebrone C, Frère J-M, Ferrer J-L, Galleni M (2011). Biochemical and Structural Characterization of the Subclass B1 Metallo-*β*-Lactamase VIM-4. Antimicrob. Agents CH., 55(3): 1248-1255.

Petrozzino JJ, Smith C, Atkinson MJ (2010). Rapid diagnostic testing for seasonal influenza: an evidence-based review and comparison with unaided clinical diagnosis. J. Emerg. Med., 39(4): 476-490.

Robert R, Pihet M (2008). Conventional methods for the diagnosis of dermatophytosis. Mycopathologia, 166(5-6): 295-306.

Sali A, Blundell TL (1993). Comparative protein modelling by satisfaction of spatial restraints. J. Mol. Biol., 234: 779-815.

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular cloning: a laboratory manual, 2nd, edn,: Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y,

Sherbrock-Cox V, Russell NJ , Gacesa P (1984). The purification and chemical characterisation of the alginate present in extracellular material produced by mucoid strains of Pseudomonas aeruginosa. Carbohydr. Res., 135, 147-154.

Tally FP, Cuchural GJ Jr, Malamy MH (1984). Mechanisms of resistance and resistance transfer in anaerobic bacteria: factors influencing antimicrobial therapy. Rev. Infect. Dis., 6 Suppl 1:S260-

269.
Tally FP, Malamy MH (1986). Resistance factors in anaerobic bacteria. Scand. J. Infect. Dis. Suppl., 49: 56-63.
Tamura K, Peterson D, Peterson N, Stecher G, Nei M, and Kumar S (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol. Biol. Evol., 28: 2731-2739.
Valenzuela JG (2002). High-throughput approaches to study salivary proteins and genes from vectors of disease. Insect. Biochem. Mol. Biol., 32(10): 1199-1209.
Venkatachalam KV, Huang W, LaRocco M, Palzkil T (1994). Characterization of TEM-1 P-Lactamase Mutants from Positions 238 to 241 with Increased Catalytic Efficiency for Ceftazidim. J. Boil. Chem., 269: 23444-43450.
Vrtis MC (2008). Is your patient taking the right antimicrobial? Am. J. Nurs., 108(6): 49-55.