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Research Article

MOLECULAR MODELING AND DOCKING OF AHEABC EFFLUX PUMP IN *AEROMONAS HYDROPHILA*

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ABSTRACT

Among the MDR efflux pump mechanisms in *Aeromonas hydrophila*, the Ahe ABC efflux pump **lays** a major role in the multiresistance mechanism. The AheB protein structure is predicted using MODELLER 9.9 and the final model is refined by energy minimization. The quality of the refined model is assessed using PROCHECK. The interaction between the efflux pump inhibitors 1-(1-naphthylmethyl)-piperazine (NMP) and phenyl-arginine-β-naphthylamide (PAβN) is analysed in silico by GOLD Software. The results of this study will provide an insight into the understanding of the mechanism of Ahe ABC efflux pump and the identification of the novel method to block the mechanism.

Keywords: *A.hydrophila*, Multidrug resistance, Efflux pump, AheB.

INTRODUCTION

Aeromonas hydrophila is a heterotrophic, Gram-negative, rod-shaped bacterium mainly found in areas with a warm climate in freshwater or brackish water[1]. *A.hydrophila* is also considered as a food-borne pathogen that is transmitted to humans via ingestion of contaminated food and water[2]. Clinical manifestations include gastroenteritis, skin and soft tissue infections, and a series of clinical syndromes that appear in immunocompromised patients[3].

A.hydrophila exhibits resistance to multiple drugs[4]. The multidrug resistance of most of the Gram-negative bacteria is mainly contributed by the expression of Multidrug resistance (MDR) efflux systems[5]. These systems simultaneously extrude the structurally-unrelated substrates including drugs in different classes[6]. The efflux pumps are transport proteins involved in the extrusion of toxic substrates (including virtually all classes of clinically relevant antibiotics) from within cells into the external environment. These proteins are found in both Gram-positive and -negative bacteria as well as in eukaryotic organisms[7]. Pumps may be specific for one substrate or may transport a range of structurally dissimilar compounds (including antibiotics of multiple classes); such pumps can be associated with multiple drug resistance (MDR).

In the prokaryotic kingdom there are five major families of **efflux** transporters[8]: MF (major facilitator), MATE (multidrug and toxic **efflux**), RND (resistance-nodulation-division), SMR (small multidrug resistance) and ABC (ATP binding cassette). All these systems utilize the proton motive force as an energy source[9] apart from the ABC family, which utilizes ATP hydrolysis to drive the export of substrates. Recent advances in DNA technology and the advent of the genomic era have led to the identification of numerous new members of the above families, and the ubiquitous nature of **efflux pumps** is remarkable.

Among the MDR efflux pump, the AheABC efflux system has been taken in this study. The AheABC efflux pump is chromosomally located and organized in an operon with the same direction[10]. The three genes namely *Aeromonas hydrophila* efflux A (AheA), *Aeromonas hydrophila* efflux B (AheB) and *Aeromonas hydrophila* efflux C (AheC) genes encode for membrane fusion protein, inner membrane transporter and outer membrane protein, respectively. The recently sequenced genome of *A.hydrophila* subsp. *hydrophila* ATCC 7966 is 4.7-Mb in size[11].The AheB (*Aeromonas hydrophila* efflux B), is most closely related to that of the major AcrB system of *Escherichia coli*. The aheA (1,194 bp) and aheC (1,434 bp) genes are located immediately upstream and downstream of aheB (3,150 bp), respectively.

The aims of the present study are to predict the 3-Dimensionl structure of *Aeromonas hydrophila* efflux B protein using Molecular Modelling. Additionally the interaction of AheB Efflux pump with the efflux pump inhibitors (EPIs) 1-(1-naphthylmethyl)-piperazine (NMP) and phenyl-arginine-β-naphthylamide (PAβN) has been studied using the Molecular Docking Analysis.

MATERIALS AND METHODS

Sequence Analysis

The primary sequences of the three proteins encoded by the AheABC Efflux pump system (Accession No's. ABU54830, ABU54831, ABU54832) of *A.hydrophila* was obtained from the public domain protein sequence database of NCBI (http://www.ncbi.nlm.nih.gov). The AheA (397 a.a) gene encodes the membrane fusion protein, has 89% similarity to the membrane-fusion protein (AcrA) of *Aeromonas salmonicida*. The AheB (1049 a.a) gene encodes the resistance nodulation cell division efflux pump (Protein export membrane protein), has 93% similarity to the AcrB protein *Aeromonas salmonicida*. The AheC (477 a.a) gene encodes the outer membrane protein, has 91% similarity to the outer membrane protein OprM *Aeromonas salmonicida*.

Structure Analysis

The Membrane export protein (AheB) is responsible for exporting the multiple drugs. The availability of the 3-Dimensional structure of AheB becomes necessary. Since the three-dimensional structure of AheB protein was not available in Protein Data Bank (PDB) (http://www.rcsb.org/pdb), the present task of developing the 3D model of AheB protein was undertaken.

Homology modeling of AheB:

To gain an insight into the structural characteristics of the AheB protein of *A.hydrophila* comparative modeling techniques has been employed. Molecular modeling, being the method of choice in the absence of experimentally determined crystal structure, can provide rationally good and accurate structural model for wide array of applications. The methodology used to derive the AheB model is partitioned into four phases: template selection, model generation, refinement and model evaluation.

Template selection

Similarity search was performed using Position specific iteration-basic local alignment search tool (PSI-BLAST)[12] against PDB database keeping default parameters like E-value threshold 10, word size 3 and Blosum 62 Matrix. Total three iterations of PSI-BLAST were considered as the BLAST search results converged after three iterations. So, high-resolution X-ray crystallography structure of the Multidrug Exporter Acrb protein from *Escherichia Coli* (PDB

ID: 2J8S: A chain) at resolution of 2.54 Å obtained from PDB was selected as a template protein showing 83% identity with the target protein.

```
 _aln.pos          10        20        30        40        50        60
2J8S      MPNFFIDRPIFAWVIAIIIMLAGGLAILKLPVAQYPTIAPPAVTISASYPGADAKTVQDTVTQVIEQN
target    MARFFIDRPIFAWVIALVIMLAGSLAIIKLPVAQYPSIAPPAVGISASYPGASAKTVEDSVTQIIEQN
 _consrvd *  *************  ***** *** ******** ****** ******** **** * *** ****

 _aln.p   70        80        90        100       110       120       130
2J8S      MNGIDNLMYMSSNSDSTGTVQITLTFESGTDADIAQVQVQNKLQLAMPLLPQEVQQQGVSVEKSSSSF
target    MTGLDHLLYMSSQSDSSGRVSVTLTFQPGTDPDIAQVQVQNKLQQAMSLLPQEVQQQGIRVQKTSSSF
 _consrvd * * * * **** *** * *  ****  *** ************ ** **********  * * ****

 _aln.pos  140       150       160       170       180       190       200
2J8S      LMVVGVINTDGTMTQEDISDYVAANMKDAISRTSGVGDVQLFGSQYAMRIWMNPNELNKFQLTPVDVI
target    LMVAAFISKDGAMTNDDLADYVVANIKEPLSRLDGVGDITLFGSQYSMRVWLDPNKLNRVQMTPGDVQ
 _consrvd ***   *  ** **  *  *** ** *   **  ****  ****** ** *  ** **  * ** **

 _aln.pos    210       220       230       240       250       260       270
2J8S      TAIKAQNAQVAAGQLGGTPPVKGQQLNASIIAQTRLTSTEEFGKILLKVNQDGSRVLLRDVAKIELGG
target    AAIKAQNTQVAFGKLGGTPAVSDQRFTATIMGQTRLSTVEEFNDILLRVNQDGSKVRLKDVARVELAG
 _consrvd  ****** *** * ***** *  *   * *  ****   ***  *** ****** * * ***  ** *

 _aln.pos      280       290       300       310       320       330       340
2J8S      ENYDIIAEFNGQPASGLGIKLATGANALDTAAAIRAELAKMEPFFPSGLKIVYPYDTTPFVKISIHEV
target    ESYDAEALYNGQSTAAVAIKLATGANALDTAEKVRGKLNELSTYFPANMEIVYPYDTTPFVKISIEEV
 _consrvd * **  *  ***      **************   *  *       **    *************** **

 _aln.pos        350       360       370       380       390       400
2J8S      VKTLVEAIILVFLVMYLFLQNFRATLIPTIAVPVVLLGTFAVLAAFGFSINTLTMFGMVLAIGLLVDD
target    VQTLVEAIFLVFCVMYLFLQNFRATLIPTIAVPVVLLGTFGVMSAFGFSINTLTMFGLVLAIGLLVDD
 _consrvd * ****** *** ***************************** *  ************** **********

 _aln.p  410       420       430       440       450       460       470
2J8S      AIVVVENVERVMAEEGLPPKEATRKSMGQIQGALVGIAMVLSAVFVPMAFFGGSTGAIYRQFSITIVS
target    AIVVVENVERLMSEEDLSPLEATRKSMTQITGALVGIALVLSAVFVPMAFFGGSTGAIYRQFSLTIVS
 _consrvd ********** * ** * * ******* ** ******* ************************* ****

 _aln.pos  480       490       500       510       520       530       540
2J8S      AMALSVLVALILTPALCATMLKPIAKGDHGEGKKGFFGWFNRMFEKSTHHYTDSVGGILRSTGRYLVL
target    AMVLSVLVALILTPALCATLLKPIKHGEFG-AKRGFFGWFNRAFDASANRYQSGVRKVVKQGVRYGII
 _consrvd ** ***************** ****  *  *  * ******** *  *    *    *          **
 _aln.pos    550       560       570       580       590       600       610
2J8S      YLIIVVGMAYLFVRLPSSFLPDEDQGVFMTMVQLPAGATQERTQKVLNEVTHYYLTKEKNNVESVFAV
target    YAAMLAVLAILFMRMPTSFLPEEDQGVIMSMVQLPVGATKQRTEVVLADMRDYFMKNEKDNVDSVLTV
 _consrvd *       * ** * * **** ***** * ***** ***  **  **      *    ** ** **  *

 _aln.pos      620       630       640       650       660       670       680
2J8S      NGFGFAGRGQNTGIAFVSLKDWADRPGEENKVEAITMRATRAFSQIKDAMVFAFNLPAIVELGTATGF
target    AGFSFAGSGQNSGMAFIKLKDWKERNTPDRSANAIIGRAMGYLFSIKEAQVFAFNLPPIPELGTATGF
 _consrvd  ** *** *** * **  ****  *        **  **      ** * ******* * ********

 _aln.pos        690       700       710       720       730       740
2J8S      DFELIDQAGLGHEKLTQARNQLLAEAAKHPDMLTSVRPNGLEDTPQFKIDIDQEKAQALGVSINDINT
target    DFFLQDRAGVGHDKLMAARNQLLGMAAQDPN-LVRVRPNGMEDTPQLDIKIDYEKALAQGLSISDINS
 _consrvd ** * * ** ** **  ******  **  *  *  ***** *****  * ** *** * * ** ***

 _aln.p  750       760       770       780       790       800       810
2J8S      TLGAAWGGSYVNDFIDRGRVKKVYVMSEAKYRMLPDDIGDWYVRAADGQMVPFSAFSSSRWEYGSPRL
target    TLSSAWGSAYVNDFVDRGRVKKVYLQADAPFRMNPEDLKLWYVRNSAGQMVPFSAFASTDWSFGSPRL
 _consrvd **  ***  ***** **********     *  ** * *   ****   ********* *  *  *****

 _aln.pos  820       830       840       850       860       870       880
2J8S      ERYNGLPSMEILGQAAPGKSTGEAMELMEQLASKLPTGVGYDWTGMSYQERLSGNQAPSLYAISLIVV
target    ERYNGVPAMEIVGEAAPGKSTGDAMAAIEQMVKQLPEGIGIEWTGLSYQERQAGSQAPALYAISLLVV
 _consrvd ***** * *** * ******** **   **    ** * *  *** *****  * *** ****** **

 _aln.pos    890       900       910       920       930       940       950
2J8S      FLCLAALYESWSIPFSVMLVVPLGVIGALLAATFRGLTNDVYFQVGLLTTIGLSAKNAILIVEFAKDL
target    FLCLAALYESWSIPFSVMLVVPLGVLGAIVAATLRGLENDVYFQVGLLTTIGLSAKNAILIVEFAKEL
 _consrvd ************************** **  *** *** ****************************** *

 _aln.pos      960       970       980       990      1000      1010      1020
2J8S      MDKEGKGLIEATLDAVRMRLRPILMTSLAFILGVMPLVISTGAGSGAQNAVGTGVMGGMVTATVLAIF
target    YDR-GMGLSEAVVEAARLRLRPILMTSLAFILGVLPLVISTGAGASSRNAIGTGVMGGMISATVLAI-
 _consrvd  *  * ** **   * * ***************** **********    ** ********  ******

 _aln.pos       1030      1040
2J8S      FVPVFFVVVRRRFSRKNEDI
target    --------------------
 _consrvd
```

Figure 1: Alignment between the target and the template sequence

Model generation

The model was generated using a comparative modeling program MODELLER 9.9[13] which generates a refined three dimensional homology model of a protein sequence based on a given sequence alignment and selected template. MODELLER employs probability density functions (PDFs) derived analytically using statistical mechanics and empirically using a database of known protein structures as the spatial restraints rather than energy. MODELLER infers distance and angle constraints from a template structure and coalesce them with energy terms for sufficient stereochemistry in an objective function which is afterward optimized in Cartesian space with conjugate gradients and molecular dynamic methods. The script "align2d.py" has been employed to perform an alignment between the target and template sequence. A rough 3D model was then obtained using the script "model-default.py" based on the generated alignment.

Refinements

The rough model generated was subjected to energy minimization using the steepest descent technique to eliminate bad contacts between protein atoms. Computations were carried out in vacuo with the GROMOS96 43B1 parameters set, implemented through Swiss-PdbViewer (http://expasy.org/spdbv/).

Evaluation

The backbone conformation of the rough model was inspected using the Phi/Psi Ramachandran plot obtained in the PROCHECK server (http://nihserver.mbi.ucla.edu/SAVES_3/saves.php). The results of Ramachandran plot indicates that the rough model generated had four residues (THR638, PRO671, THR677, and SER754) in the disallowed region, occurring in the loop.

Loop refinement and evaluation

The rough model has been subjected to loop refining using "loop.py" script. The obtained model was subjected to energy minimization. The Ramachandran plot of the final model had no residues in the disallowed region.

Molecular Docking

Active Site Prediction

After obtaining the final model, the possible binding sites of AheB protein were searched using Q-SiteFinder (http://bmbpcu36.leeds.ac.uk/qsitefinder/). Ten binding sites were obtained for AheB from Q-SiteFinder. The active site of the protein includes LEU357, PHE358, LEU359, GLN360, GLU414, GLU417, ARG418, SER421, GLU422, ILE500, GLU504, PHE505, GLY506, PHE515, ASN516, ARG517, PHE519, ASP520, ALA523, ARG966, LEU967, ARG968, LEU969, ARG970 and PRO971.

Inhibitors of Efflux Pump

1-(1-naphthylmethyl)-piperazine (NMP) and phenyl-arginine-β-naphthylamide (PAβN) are potential inhibitors of resistance-nodulation-division (RND) family efflux systems[14]. The 2D structures of NMP and PAβN were drawn using ACD Chemsketch (www.acdlabs.com/). The structures were then converted to 3D, their geometries were optimized and saved in "MDL mol file" format.

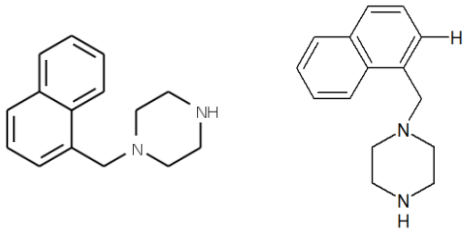

Figure 2: Structure of 1-(1-naphthylmethyl)-piperazine (NMP) and phenyl-arginine-β-naphthylamide (PAβN)

GOLD Docking Simulations

The two efflux pump inhibitors 1-(1-naphthylmethyl)-piperazine (NMP) and phenyl-arginine-β-naphthylamide (PAβN) were docked in to the binding site of the receptor AheB protein model using GOLD (Genetic Optimization of Ligand Docking) software provided by CCDC,U.K[15]. The GOLD program uses a genetic algorithm (GA) to explore the full range of ligand conformational flexibility and the rotational flexibility of selected receptor hydrogens. Grid was prepared for the protein with the center and the size of the bounding box set on 10 Å. The coordinates of the enclosing box (x = 121 Å; y = 87 Å; z = 45 Å) were defined starting from the set of active site residues.

RESULTS

Homology modeling of AheB

The absence of the three dimensional structure for AheB of *A.hydrophila* in PDB prompted us to construct the 3D model. The three dimensional structure provides valuable insight into the mechanism of multidrug resistance. Among the three conformations generated, the one with the least modeller objective function value was considered to be thermodynamically stable and chosen for

further refinement and validation. The stereochemistry of the constructed model was subjected to energy minimization and the stereochemical quality of the predicted structure was assessed. The Ramachandran plot for the model showed 94.6% of the residues in the core region, 4.9% residues in the allowed regions, 0.2% in the generously allowed regions and 0.3% residues in the disallowed region. Therefore there are four residues present in the stereochemically unstable regions. Hence, these residues were subjected to refinement and further energy minimization. In an analysis of the final model, 94.9% of the residues were found to occupy the core region. The residues in the disallowed region had been shifted to the allowed region, thereby optimizing and stabilizing the overall conformation of the predicted structure.

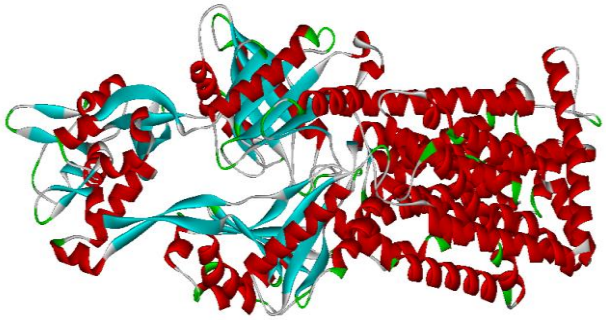

Figure 3: Three Dimensional structure of AheB protein

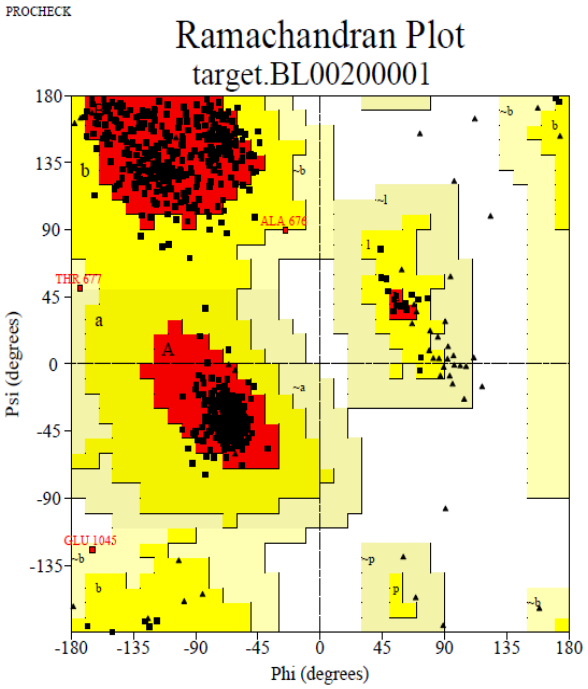

Figure 4: Ramachandran plot of AheB protein built using MODELLER software.

Molecular Docking

Docking of all the two major efflux pump inhibitors with the AheB protein was performed using the GOLD software resulted in identifying the best compound that interact with the receptor. The software generated 10 different conformations of each compound used for the study. The results were evaluated based on the binding compatibility i.e. Docked energy in kcal/mol. The 53 amino acid active site pocket provides a cavity for the active plant compounds to interact with the receptor. The efflux pump inhibitor binds with the AheB protein with the highest GOLD score of 33.81, whereas phenyl-arginine-β-naphthylamide (PAβN) binds with a GOLD score of 31.56.

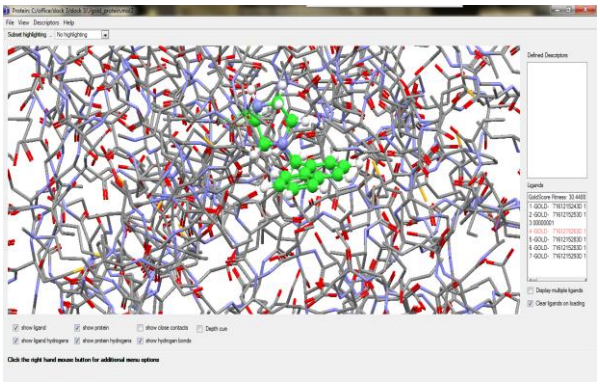

Figure 5: Interactions between 1-(1-naphthylmethyl)-piperazine (NMP)

From the results of the hydrogen bond formation between the active site of AheB protein and the efflux pump inhibitors it is evident that NMP forms 2 hydrogen bonds whereas PAβN doesn't form any bonds, hence has only very weak interactions.

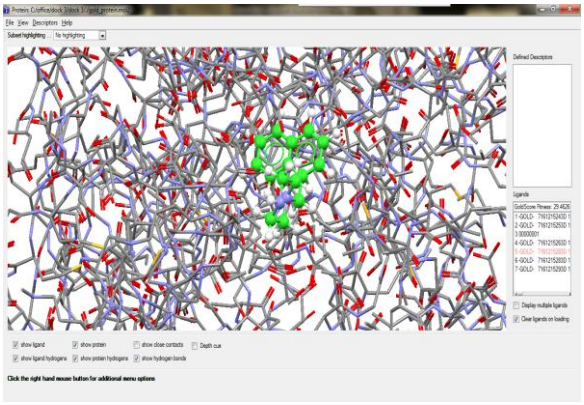

Figure 6: Interactions between phenyl-arginine-β-naphthylamide (PAβN) and AheB Protein

DISCUSSION

Mounting concerns for emergence of drug- resistance among aeromonads are reflected in a number of reports[16-19]. The problems of multi-drug resistant aeromonads are more intricate in developing nations like India and other South East Asian countries. Among gram-negative bacteria, many of these MDR efflux pumps belong to the RND (resistance-nodulation-cell division) type family of tripartite efflux pumps. The present work was established to study in detail about the AheABC Efflux pump in *Aeromonas hydrophila* which plays a predominant role in the multidrug resistance mechanism. Initially the absence of the tertiary structure of the AheB protein interested us to predict the **structure** using MODELLER9.9, the resulting model was with a high quality as proved by the Ramachandran plot evaluations. Further to the tertiary structure prediction the efflux pump inhibitors were docked with the AheB protein model to study the interaction between them. The result shows that NMP binds with a higher score with AheB when compared to PAβN. The present work will be a platform for performing further more studies on the development of a novel inhibitor for the Efflux pump mechanisms.

REFERENCES

1. Abbott SL.Aeromonas. In: PR Murray, EJ Baron, MA Pfaller, JH Jorgensen and RH Yolken, editors.Manual of clinical microbiology, 8th ed. Washington, DC, AMS Press;2003. p. 701–705.
2. Daskalov H.The importance of Aeromonas hydrophila in food safety. Food Cont.2006; 17(6): 474-483.
3. Janda JM and Abbott SL.Evolving concepts regarding the genus Aeromonas: an expanding panorama of species, disease

presentations, and unanswered questions. Clin. Infect. Dis.1998; 27:332–344.

4. Son R, Rusul G, Sahilah AM, Zainuri A, Raha AR and Salmah I. Antibiotic resistance and plasmid profile of Aeromonas hydrophila isolates from cultured fish, Telapia (*Telapia mossambica*). Lett Appl Microbiol.1997; 24(6): 479-482.
5. Nikaido H.Multidrug efflux pumps of Gramnegative bacteria. J Bacteriol.1996; 178:5853-5859.
6. Poole K.Efflux mediated multidrug resistance in Gram Negative Bacteria. Clin.Microbiol. Infec.2004; 10(1):12-26
7. Bambeke VF, Balzi E and Tulkens PM.Antibiotic efflux pumps. Biochem.Pharmacol.2000; 60:457–470.
8. Lomovskaya O, Warren MS, Lee A, Galazzo J, Fronko R, Lee M. *et al*. Identification and characterization of inhibitors of multidrug resistance efflux pumps in *Pseudomonas aeruginosa*: novel agents for combination therapy. Antimicrob. Agents Ch. 2001; 45: 105–116
9. Paulsen IT, Brown MH and Skurray RA.Proton-dependent multidrug efflux systems. Microbiol. Rev. 1996; 60: 575–608
10. Hernould M., Gagne S, Fournier M, Quentin C and Arpin C. Role of the AheABC efflux pump in Aeromonas hydrophila intrinsicmultidrug resistance. Antimicrob. Agents Ch.2008; 52(4): 1559-1563.
11. Seshadri R, Joseph SW, Chopra AK, Sha J, Shaw J,Graf J *et al*. Genome sequence of Aeromonas hydrophila ATCC 7966T: jack of all trades. J. Bacteriol. 2006;188:8272–8282.
12. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. A basic local alignment search tool. J. Mo.l Biol.1990; 215: 403–10.
13. Sali A, Blundell TL.Comparative protein modelling by satisfaction of spatial restraints. J. Mol. Biol. 1993; 234(3): 779–815.
14. Xiaowen RB, Julie AP and James EB. Effect of the efflux inhibitors 1-(1-naphthylmethyl)-piperazine and phenyl-arginine-b-naphthylamide on antimicrobial susceptibility and virulence factor production in Vibrio cholera. J. of Antimicrob. Chemoth. 2009; 63: 103–108
15. Jones G,Willett P,Glen RC, Leach AR and Taylor R.Development and Validation of Genetic Algorithm for flexible docking. J.Mol.Biol.1997; 267:727-748
16. Zheng G, Zhou K, Zheng GX and Zhou K.Drug resistance of Aeromonas hydrophila strainsisolated from skin ulcer of *Anguilla anguilla*. Journal of Fishery Sciences of China 1999; 6:69-72.
17. Chandrakanthi WHS, Pathiratne A and Widanapathirana GS. Characteristics andvirulence of Aeromonas hydrophila isolates fromfreshwater fish with Epizootic Ulcerative Syndrome (EUS).Diagn.Microbiol. Infect.Dis. 2000; 28: 29-42.
18. Yucel N and Ctak S.The occurrence, hemolytic activity and antibiotic susceptibility of motileAeromonas spp. isolated from meat and milk samplesin Turkey.J.Food Safety 2003; 23:189-200.
19. Emekdas G, Aslan G, Tezcan S, Serin MS, Yildiz C, Ozturhan H and Durmaz R.Detection ofthe frequency, antimicrobial susceptibility andgenotypic discrimination of Aeromonas strains isolatedfrom municipally treated tap water samples bycultivation and AP-PCR in turkey. *Int.* J.Food Microbiol.2006; 107:310-314.