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Review Article
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PROTEIN IDENTIFICATION OF AMINOGLYCOSIDES RESISTANT MYCOBACTERIUM TUBERCULOSIS BY MALDI-TOF MS AND BIOINFORMATICS TOOLS

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ABSTRACT:

Tuberculosis (TB) is the major cause of death worldwide. The enormity of the problem has been further worsened by the emergence of multi-drug resistant (MDR) strains and the dual infection with HIV & diabetes. A newly identified TB threat which leaves patients virtually untreatable using currently available anti-TB drugs is XDR-TB. Aminoglycosides are commonly used in the tuberculosis treatment and are drug of choice especially for the category II patient. They inhibit protein synthesis in susceptible bacteria by interacting with several steps of the translationGenomic studies are also helpful to understand the resistance mechanisms, but still our knowledge is incomplete. In any cell, proteins are ultimately working moiety, therefore proteomic analysis of mycobacteria in relation to aminoglycosides resistance could be much informative. The aim of the present study was to analyze and compare the lipophilic proteins of total sensitive and aminoglycosides resistant M. tuberculosis clinical isolates. On analyzing 2D gels, six proteins were found to be over expressed and were identified using MALDITOF MS. These proteins were further characterized by bioinformatics tools such as TBPred, DAS, phylodendron, I-tasser, patchdock, firedock etc.

Keywords: Aminoglycosides, Resistant, Mycobacterium tuberculosis, Maldi-Tof Ms And Bioinformatics Tool

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1. INTRODUCTION:

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis*, an important member of the genus *Mycobacterium.* It has been called the 'white plague' and 'the captain of all men of death' **(Ananthnarayan et al., 2001).** It is also described with different names 'king's evil' 'Pthisis', 'Rajakshma' and 'Tapedic'. The causative agent of tuberculosis is *Mycobacterium tuberculosis*.

Multi-drug-resistant tuberculosis (MDR-TB) is defined as resistance to the two most effective first-line TB drugs: rifampicin and isoniazid. Extensively drug-resistant TB (XDR-TB) is an advanced form of MDR-TB with further resistance for at least one fluroquinolone and one injectable drug (Kanamycin/Amikacin or Hygromycin). Totally drug-resistant tuberculosis (TDR-TB) is a generic term for tuberculosis strains that are resistant to a wider range of drugs than strains classified as extensively drug-resistant tuberculosis.

Aminoglycosides are highly potent, broad-spectrum antibiotics with many desirable properties for the treatment of life-threatening infections **(Gilbert, 1995).** Aminoglycosides have several potential antibiotic mechanisms, some as protein synthesis inhibitors. They kill bacteria by inhibiting protein synthesis via binding to the 16S rRNA and by disrupting the bacterial cell membrane integrity **(Shakil et al., 2008).** Streptomycin (SM) is first line anti-tuberculosis drug and preferred for treatment of relapses. It inhibits protein synthesis in susceptible bacteria by interacting with steps of translation.

Ultimately proteins are the working moiety, studying the changes in the protein profile of resistant bacteria in comparison to susceptible ones may provide some fruitful information. Proteomic studies of *M. tuberculosis* and other organisms mainly involves two-dimensional gel electrophoresis (2DGE) to resolve proteins and their identification by mass spectrometry (MS) **(Mehaffy et al., 2010**). Proteomic analysis involving a combination of electrophoresis (1-DE and 2-DE), mass spectrometry and bioinformatics, has now emerged as robust and efficient strategy for rapid identification of proteins and is aided by the availability of total genome sequence database. Extracted proteins were separated in parallel by 1-D SDS-PAGE and 2-DE and then analyzed by LC-MS/MS and MALDI-MS/MS. Identified proteins were characterized with regard to biological

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functions and physicochemical properties including numbers of predicted TMD (alpha-helical transmembrane domain) and hydropathy **(Mattow et al., 2007).**

2. OBJECTIVES

In the light of above introduction following objectives have been proposed:

1. Selection of isolates of *Mycobacterium tuberculosis* showing resistance to aminoglycosides drugs.
2. Investigate the proteomic changes in the protein profiles of selected isolates by two dimensional gel electrophoresis.
3. Identification of over expressed proteins by MALDI-TOF mass spectrometry and bioinformatics tools.

3. METARIALS & METHODS

3.1 Selection of clinical isolates and culture

Aminoglycosides resistant (resistant to streptomycin, amikacin and kanamycin) and sensitive (sensitive to rifampicin, isoniazid, ethambutol, and pyrazinamide) mycobacterial clinical isolate were collected from the Mycobacterial Repository Centre, NJIL&OMD, Agra. The selected mycobacterial clinical isolates were grown in Sauton's liquid media and were incubated at 370C. After four weeks cultures were harvested.

3.2Preparation of whole cell lysate and phase partition

Whole cell lysate (WCL) was prepared according to the recommended protocol using sonication and differential centrifugation. Triton X-114 was added to the WCL (final detergent concentration 2%, v/v) and the suspension was stirred at 4°C for 20 min. to obtain the protein extract in a single phase. Residual insoluble matter was removed by centrifugation at 15700g for 10 min, and the solution separated in two phases, an upper (aqueous) and a lower (detergent) phase after 10 min incubation at 37°C. Both phases were collected.

3.3 Protein precipitation with SDS-TCA-Acetone

Detergent phase and aqueous phase was precipitated using acetone and SDS-TCA-Acetone method (**Bisht et al., 2007**). The protein pellet was dissolved in appropriate volume of 2D rehydration buffer and the protein concentration was estimated using the Bradford assay.

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3.4 Protein Estimation

Proteins were estimated by the method of Bradford **(Bradford, 1976)** using bovine serum albumin as the standard.

3.5Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE):

Method described by Laemmli **(Laemmli, 1970)** was followed. Glass plates of mini vertical gel electrophoresis apparatus were washed, fixed in a stand and the plates were sealed. Separating gel was poured in between the plates and thin layer of d/w was added on the top of gel. Then stacking gel was added and immediately a comb was inserted. After polymerization the comb was removed and samples were loaded in the wells. For molecular weight identification, marker was used and gel was run in the running buffer at constant current (25 mA) till the bromophenol blue reached the bottom of the gel. After electrophoretic run the gel was washed and transferred to coomassie stain for overnight and then de-stained with distaining solution next day, bands of proteins will appear on gels which were then photographed.

3.6 Two-Dimensional Gel Electrophoresis

2D-PAGE was carried out using the method of "in gel rehydration" **(Gorg, et al., 2000)** with slight modifications. Cell extract (500µg protein) mixed with rehydration buffer (final vol. 300µl) was applied to immobilized (IPG) strip (17cm length, pH 4-7) and was left overnight for passive rehydration at 20ºC. IEF was performed at 20°C in an IEF protean cell using the following 5-step programme:-

1. 250 V for 2h, linear mode
2. 250 V for 2h, rapid mode
3. 5000 V for 4h, linear mode
4. 5000 V for 35000 V h, rapid mode
5. 500 V for 10h, slow mode

The current limit was set at 50µA/strip. After IEF, IPG strips were equilibrated sequentially in equilibration buffer I and II. Later, strip was loaded on the top of a vertical SDS–polyacrylamide gel (12% gel) & sealed in place with 1% low melting agarose dissolved in electrode buffer. Molecular mass markers were loaded in a separate well by the side of the strip. Electrophoresis was performed using Laemmli's **(1970)** buffer system at a constant current of 25mA until the indicator dye (bromophenol blue) approached the bottom edge of the gel. Proteins were stained by coomassie blue & then photographed.

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3.7In-Gel Digestion of Selected Protein Spots (Coomassie Stained) with Trypsin (Shevchenko et al., 1996)

Excision of Protein Bands from Polyacrylamide Gels:

Wash the gel with water (2 changes, 10 minutes each).Excise the spots of interest from the gel. Cut the excised piece into 1 mm-cubes.Transfer to a 0.5 µl microfuge tube.

Washing of Gel Pieces:

Wash the gel particles with water and 50 mM NH4HCO3/acetonitrile 1+1 (v/v) for 15 min

Remove all remaining liquid and add enough acetonitrile to cover the gel particles. The gel plugs shrink and stick together. Remove the acetonitrile. Rehydrate the gel pieces in 50 mM NH4HCO3. After 5 min add an equal volume of acetonitrile.Remove all liquid after 15 min of incubation.Add enough acetonitrile to cover the gel particles. After the gel pieces have shrunk remove the acetonitrile. Dry down gel particles in air or in vacuum centrifuge.

Reduction and Alkylation:

Swell the gel particles in 10 mM dithiotreitol/50 mM NH4HCO3 (freshly prepared).Incubate for 45 min at 56°C. Chill tubes to room temperature. Remove excess liquid and replace it quickly by roughly the same volume as above of freshly prepared 55 mM iodoacetamide (light sensitive!) in 50 mM NH4HCO3. Incubate for 30 min at room temperature in the dark.Remove iodoacetamide solution.

Wash the gel particles with 50 mM NH4HCO3 and acetonitrile (1+1; v/v), one or two changes each, 15 min per change. Add enough acetonitrile to cover the gel particles. After the gel pieces have shrunk remove the acetonitrile.Dry down the gel particles in air or in vacuum centrifuge.

3.8 Mass Spectrometric Peptide Analysis

The proteolytic masses obtained were then evaluated using Mascot, a peptide mass fingerprinting tool, which tries to determine protein from existing database. Peak detection in MALDI spectra and submission of peak lists to the Peptide mass fingerprint (PMF) Mascot server were done using the Mascot Wizard program (Matrix Science). Peptide mass tolerance was set to 100 with carbamidomethyl-cystein set as fixed modification, oxidation of methionine as variable modification and 1 missed cleavage site allowed.

3.9 Bioinformatics analysis of identified proteins:

a) **TBpred**is a prediction server that predicts four sub cellular localization (cytoplasmic,

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integral membrane secretory and membrane attached by lipid anchor) of mycobacterial proteins.

b) **Homology modeling:** The sequence of the newly identified protein was obtained from TB databases such as Tuberculist web server. Homology modeling was done with the help of modeling programs such as Swiss Model / Discovery Studio.

c) **TransMembrane prediction using DAS:** By using "DAS" - Transmembrane Prediction server transmembrane was predicted.

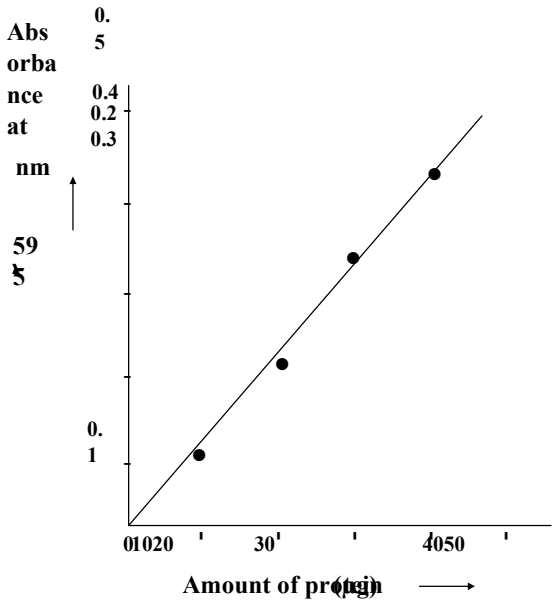
d) **Protein-drug interactions:** Homology model(s) thus prepared was used to study protein-drug interactions. This was done by docking of aminoglycoside with the secondary structure (homology model) of the newly identified protein. Docking was done by docking programs such as Autodock / Patchdoc / Discovery Studio. Docking with highest score and minimum energy was considered as final. This was helpful in explaining the mechanism that why aminoglycoside drug(s) interacts preferentially with this protein.

e) **Comparative genomics:** The role of newly identified protein was investigated by comparing its sequence with homologous sequences in other bacteria by comparative genomic approaches. It was done by performing BLASTp and multiple sequence alignment (MSA) using NCBI-BLAST server and ClustalW.

f) **Phylogenetic analysis:** The phylogenetic analysis of the newly identified protein was helpful in revealing its relationship with proteins of similar functions among other mycobacteria as well as among important bacterial pathogens. This was done by using programes like Phylip / Phylodendron etc.

4. RESULTS

4. 1. Protein Estimation



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4.2.Mass Spectrometric Analysis

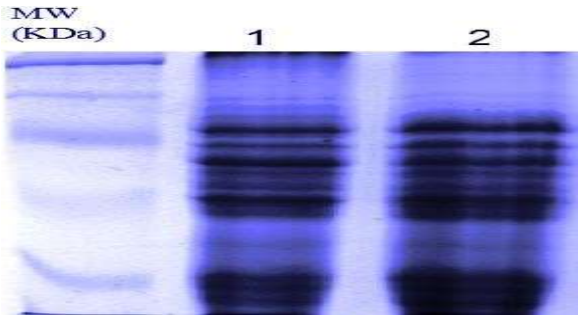

Fig 4.2 SDS-PAGE gel profile of lipophilic protein of sensitive and aminoglycoside resistant *M. tuberculosis* clinical isolateLane 1- Molecular weight marker.Lane 2- Aminoglycoside resistant isolate. Lane 3- Total sensitive isolate.

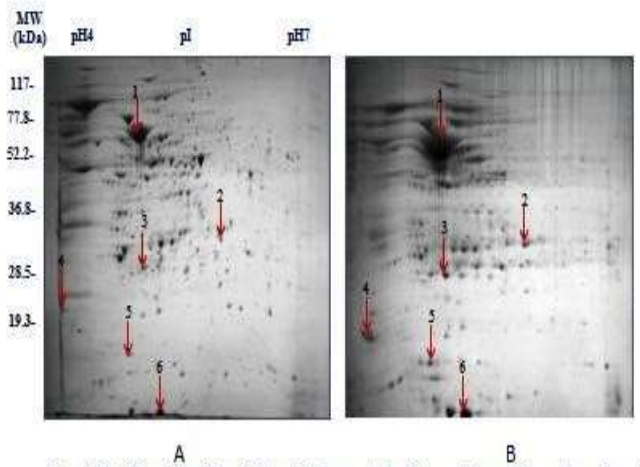

Fig. 4.3. 2D gel profile of lipophilic protein of sensitive and aminoglycoside resistant *M. tuberculosis* clinical isolates .

Table: 4.1 Details of proteins identified by mass spectrometry

Spot No.	Protein identified	MASCOT Score	Nominal Mass (Da)	pI	Sequence Coverage %	Accession Number	Function
1.	60 kDa chaperonin 2,	**85**	**56690**	**4.85**	**27%**	Rv0440	Prevents misfolding and promotes the refolding and proper assembly of unfolded polypeptides generated under stress conditions.
2.	Universal stress protein Rv2623 /MT2698	**162**	**31747**	**5.46**	**57%**	Rv2623 /MT2698	Function unknown
3.	Methoxy mycolic acid synthase	**74**	33527	**5.27**	36%	Rv0643c	Involved mycolic acid modifications
4.	Hypothetical protein	**77**	19930	**3.91**	44%	Rv3867	Function unknown
5.	Not identified	……	……	….	……	…….	……..
6.	14 kDa antigen (16 kDa antigen) (HSP 16.3)	76	16217	5.00	57%	Rv2031c	Stress protein induced by anoxia. Have a proposed role in maintenance of long-term viability during latent, asymptomatic infections and a proposed role in replication during initial infection.

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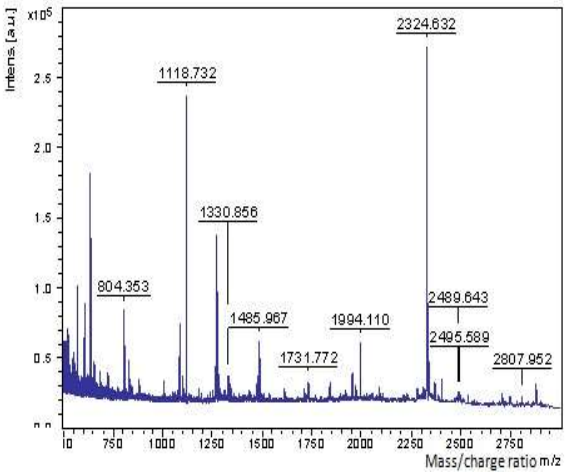

Fig.4.3: Representative peptide mass fingerprint

4.4: Bioinformatic Analysis

Bioinformatic analysis was carried out of two proteins which were not functionally identified by MALDI-TOF MS. These are Universal stress protein Rv2623 and hypothetical protein Rv3867.

4.4.1 Prediction of sub cellular localization:

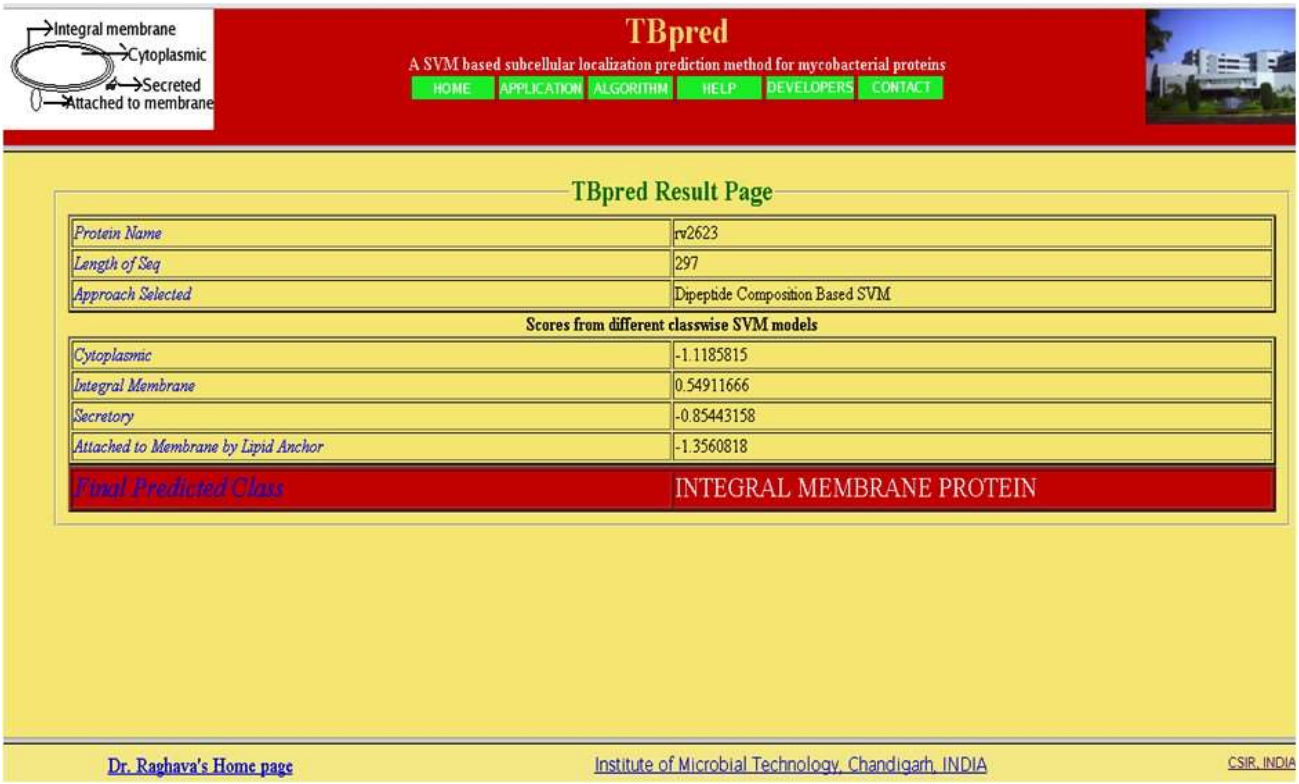

Fig. 4.4.1 Prediction of sub cellular localization of Rv2623 by dipeptide composition based model

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4.4.2 Phylogenetic trees prediction:

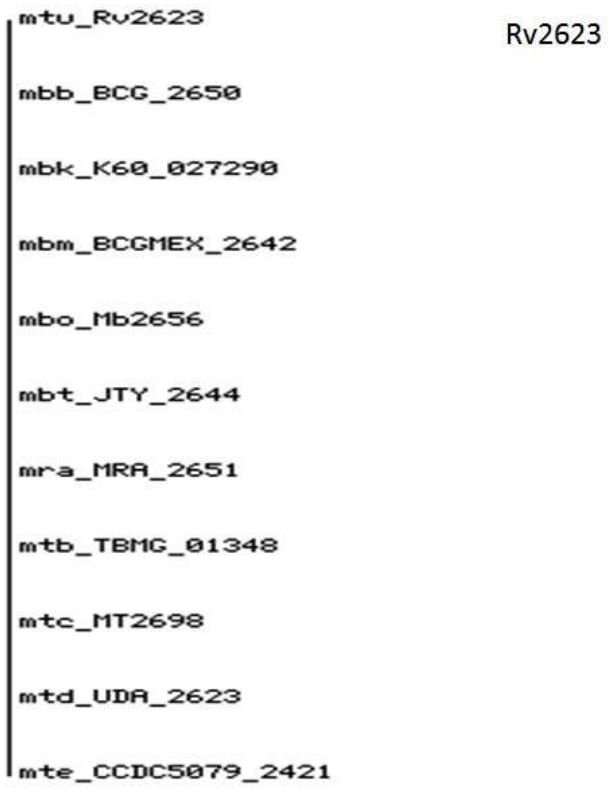

Fig. 4.4.2a: Phylogenetic tree of Rv3867

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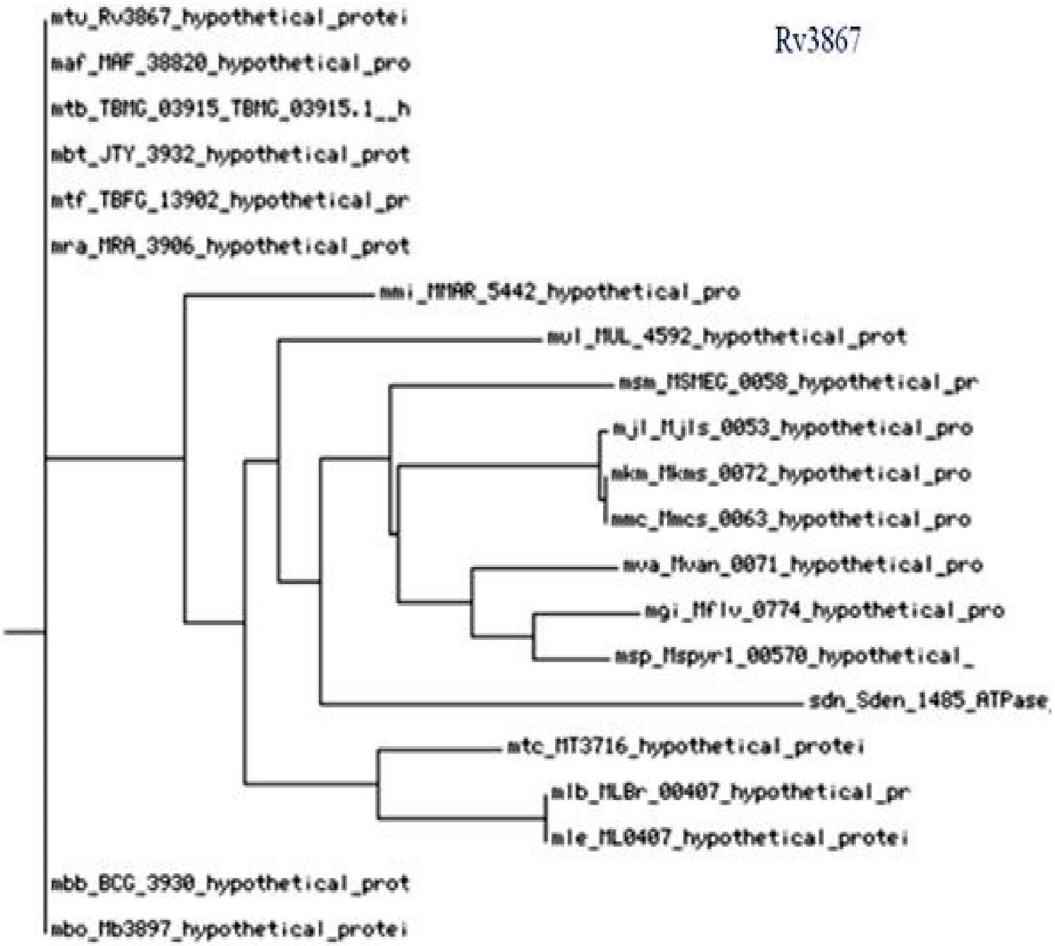

Fig. 4.4.2b: Phylogenetic tree of Rv2623

4.4.3 Transmembrane prediction:

Fig. 4.4.3 showed that Rv2623 has single transmembrane helix from amino acid 112-119.

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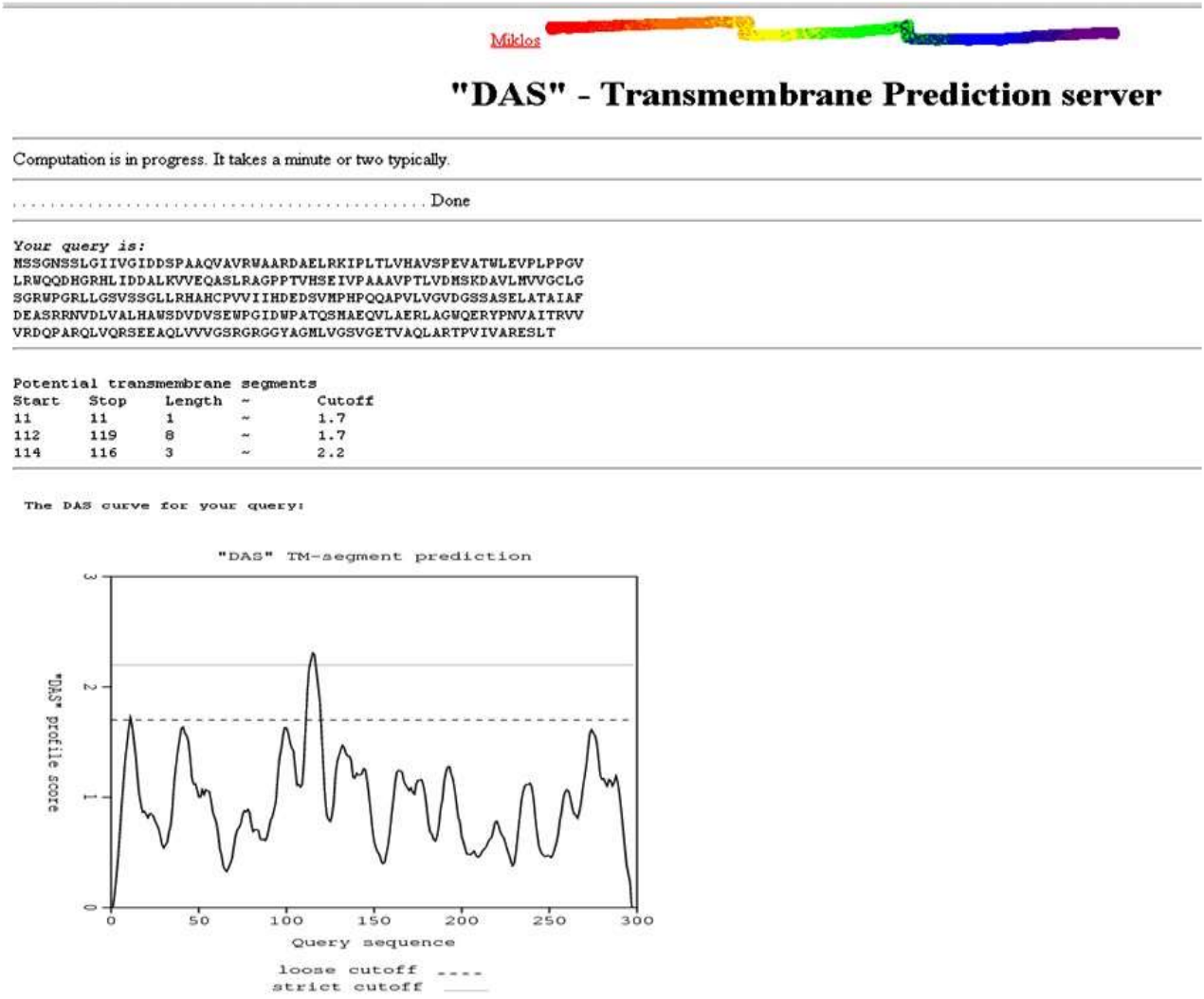

Fig. 4.4.3: Transmembrane prediction by DAS server

4.4.4 Molecular Docking:

Molecular docking of SM, AK and KM with secondary structures of Rv3867 showed successful binding of SM into the central cavity of the protein and AK, KM on the surface of protein.

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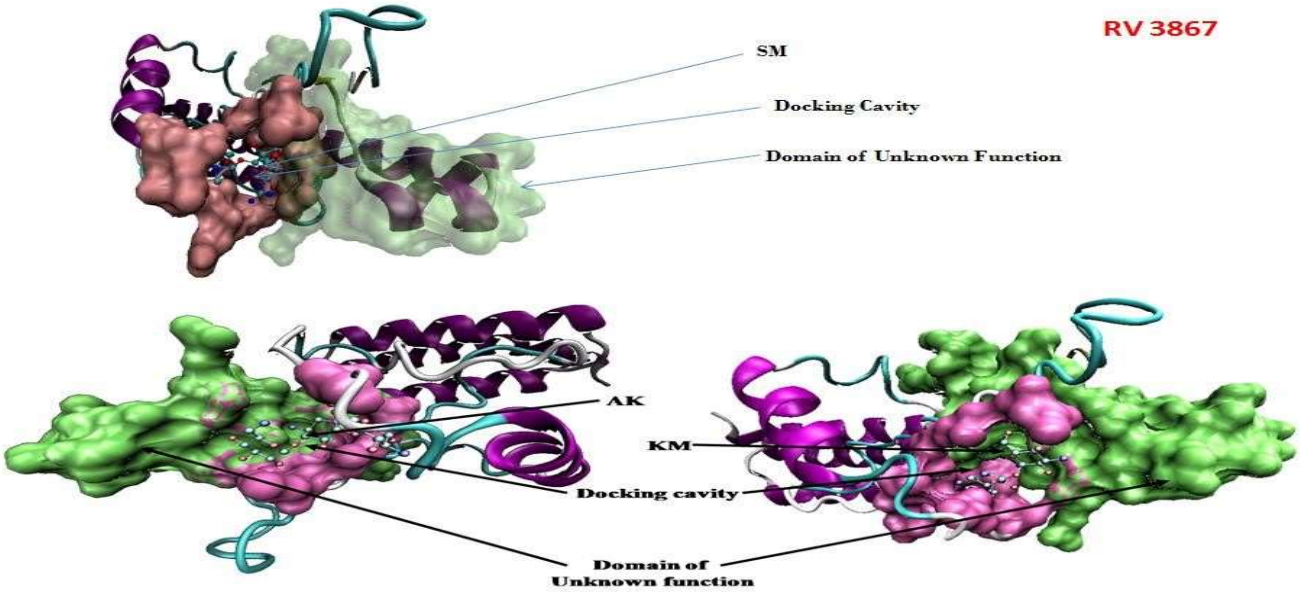

Fig.4.4.4: Molecular docking of Rv3867 with Streptomycin, Amikacin and Kanamycin

5. DISCUSSION &CONCLUSION

Our findings clearly indicated that lipophilic proteins might play an important role inresistance directly or indirectly.To conclude, this study has demonstrated the usefulness of proteomic approach to identity overexpressed proteins from lipophilic fraction of aminoglycoside resistant *M. tuberculosis* isolates compared to susceptible isolates. These investigations are expected to throw light on the expression of some novel proteins related to aminoglycoside resistance. Five spots were identified by MALDI-TOF while one spot could not be identified. Spot 1 was identified as 60 kDa chaperonin protein which prevents misfolding and promotes the refolding and proper assembly of unfolded polypeptides generated under stress conditions in *M. tuberculosis.* Spot 2 was identified as Universal stress protein Rv2623/MT2698 with unknown function. Spot 3 was identified as Methoxy mycolic acid synthase, which isinvolved in mycolic acids modification and catalyzes unusual Sadenosyl-methionine-dependent transformation of a cis-olefin mycolic acid into a secondary alcohol. Spot 4 was a hypothetical protein with an unknown function. Spot 5 was not identified. Spot 6 was 14 kDa antigen (HSP

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16.3) which is stress protein induced by anoxia and have a proposed role in maintenance of long-term viability during latent, asymptomatic infections and a proposed role in replication during initial infection.

To conclude, this study has demonstrated the usefulness of proteomic approach to identity overexpressed proteins from lipophilic fraction of aminoglycoside resistant *M. tuberculosis* isolates compared to susceptible isolates. These investigations are expected to throw light on the expression of some novel proteins related to aminoglycoside resistance.

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