Drug target identification using gene expression microarray data of *Toxoplasma gondii*

Budhayash Gautam budhayashgautam@gmail.com
Department of Computational Biology and Bioinformatics,
Sam Higginbottom Institute of Agricultural, Technology and Sciences,
Allahabad-211007, U.P., India

Pramod Katara katarapramod@gmail.com
Department of Bioscience and Biotechnology,
Banasthali University,
Rajasthan-304022, India

Satendra Singh satendralike@gmail.com
Department of Computational Biology and Bioinformatics,
Sam Higginbottom Institute of Agricultural, Technology and Sciences,
Allahabad-211007, U.P., India

Rohit Farmer rohit.farmer@gmail.com
Department of Computational Biology and Bioinformatics,
Sam Higginbottom Institute of Agricultural, Technology and Sciences,
Allahabad-211007, U.P., India

Abstract

Toxoplasma gondii is an obligate intracellular Apicomplexan parasite that can infect a wide range of warmblooded animals including humans. This pathogen is one of the most common in humans due to many contributing factors that include: (1) its complex life cycle allows it to be transmitted both sexually via felid fecal matter and asexually via carnivorism. (2) *Toxoplasma* has an extremely wide host cell tropism that includes most nucleated cells. (3) In humans and other intermediate hosts, *Toxoplasma* develops into a chronic infection that cannot be eliminated by the host's immune response or by currently used drugs. In most cases, chronic infections are largely asymptomatic unless the host becomes immune compromised and often suffer from life threatening encephalitis. Unfortunately, owing to the toxic side effects and general low efficacy of all known drugs for toxoplasmosis, new chemotherapeutic agents are urgently required. The mechanisms by which *Toxoplasma* grows within its host cell, encysts, and interacts with the host's immune system are important questions. The use of DNA microarrays in transcriptional profiling, genotyping, and epigenetic experiments has impacted our understanding of these processes. In the past years, many existing data analysis methods from other fields have been applied to gene expression data. Among these, clustering methods are a large family of commonly used data analysis methods. Clustering methods are particularly useful in the analysis of gene expression data and organization of gene expression data. In present work Hierarchical and kmeans clustering methods have been used for the analysis of microarray gene expression data of

Toxoplasma gondii with the help of Genesis tool. On the basis of coexpression, some probable gene targets in *Toxoplasma gondii* have been found (IL-16 and Inhibitor of kappa light chain Epsilon etc.). Their promoters have been predicted with Neural Network Promoter Predictor, of BDGP. Predicted promoters were aligned using ClustalW to SAG family members, (which are related to the invasion or surface attachment of the parasite to the host cell) to see common regulation. They showed good amount of sequence similarity with the already known targeted genes of SAG family. These probable targets have important functions in the case of encephalitis in AIDS patient caused by *Toxoplasma gondii*, as these targets play major role in prevention of AIDS.

Keywords: *Toxoplasma gondii*, Toxoplasmosis, Microarray Data Analysis, Hierarchical Clustering, K-means Clustering, Co-expression, Encephalitis.

1. INTRODUCTION

Toxoplasma gondii, represent a major source of human and animal disease worldwide. *T. gondii* is the cause of significant morbidity and mortality in patients who have AIDS (acquired immunodeficiency syndrome) and serious congenital birth defects in both humans and livestock. **[1]** Although infection is usually asymptomatic in healthy individuals, immune-compromised patients often suffer from life-threatening encephalitis. A common requirement for intracellular pathogens is they must scavenge nutrients from their hosts while avoiding innate host defense mechanisms **[2]**. *Toxoplasma* is no different and how it replicates within a host cell has been the focus of intense investigation by several laboratories. Biochemical- and cell-biological-based assays demonstrated that parasites modify host microtubule and intermediate filament organization **[3, 4, 5]**, inhibit host cell apoptosis **[6, 7]**, upregulate pro-inflammatory cytokines **[8, 9, 10, 11]**, and scavenge purine nucleosides, cholesterol, and other nutrients from their host cells **[12, 13]**. To examine the molecular basis for these changes, DNA microarrays have been used to analyze changes in host gene expression following infection **[14, 15, 16]**. These studies indicated that changes in host transcription were extremely widespread. These changes came in at least two distinct waves with the first wave being induced within 2 hours and included a large number of pro-inflammatory response genes **[15]**. The second wave of gene expression included genes that encode proteins that function in a diverse set of cellular processes. Most striking from these studies was the finding that glucose, mevalonate, and iron metabolic genes were upregulated specifically by *Toxoplasma* **[15]**.

An important advantage of DNA microarrays is that they can readily examine multiple time points and conditions **[17].** As a first step, microarrays spotted with the cDNAs used for the bradyzoite EST sequencing project **[18]** were generated and used to compare the transcriptional responses that take place at various time points following induction of differentiation **[19]**. Although these first generation microarrays were spotted with fewer than 650 unique genes, they demonstrated that the microarrays could be used to discover additional bradyzoite-specific genes. Besides gene discovery, DNA microarrays can also be used to map transcriptional pathways. As an example, the transcriptional response of wild-type parasites and bradyzoite differentiation mutants were compared after stimulating the parasites to undergo differentiation. The resulting microarray data demonstrated that the transcriptional pathways induced during development were hierarchal **[20, 21]**. The full complexity associated with differentiation was demonstrated using full-genome *Toxoplasma* microarrays that compared the transcriptional responses of three distinct *Toxoplasma* strains to a drug that induces bradyzoite development **[22].** GRA (dense granule proteins) and SAG (Surface antigens) are known to have some importance in the cavenging of nutrients from their hosts and invasion to their host's cell respectively **[21]**.

As a parasite with a potentially devastating clinical outcome, an important goal of toxoplasmosis research is the development of new drugs and treatments. There are two major reasons that new drugs are needed to treat *Toxoplasma* infections. First, the drugs currently used to treat *Toxoplasma* infections are poorly tolerated, have severe side effects, and cannot act against bradyzoites **[23]**. Second, there are reports that *Toxoplasma* is developing resistance to the current generation of drugs **[24, 25]**. How resistance to these drugs has developed is not known but is critical to understand because it will lead to improved drug design and will increase our understanding of the biological functions of these drug targets. One way to understanding mechanisms of resistance is to compare the transcriptional profiles of wild-type and resistant parasites grown in the absence or presence of the drug. Such studies in bacterial resistance have demonstrated that pathogen responses to antibiotics are multifactorial and complex **[26].** Whether the same will be true in *Toxoplasma* is unclear, but data from these types of experiments will likely impact new anti-*Toxoplasma* drug design.

Possible drug targets can be detected with the help of microarray data analysis **[27, 28]**. Microarray technology can either be used to investigate the functions of genes, or be used in the diagnosis of diseases **[29]**. In the past years, many existing data analysis methods from other fields have been applied to gene expression data; also many novel methods are developed or under developing particularly for gene expression data analysis **[30, 31]**. Among these, clustering methods are a large family of commonly used data analysis methods. Clustering methods are particularly useful in the analysis of gene expression data and organization of gene expression data and the results from clustering can be used as a starting point for further analysis. A natural basis for organizing gene expression data is to group together genes with similar patterns of expression i.e. co-expressed genes. Co-expressed genes may reveal much about coregulatory mechanisms. For example, if a single regulatory system controls two genes, then the genes are expected to be co-expressed. In general there is likely to be a relationship between co-expression and co-regulation. In co-expression analysis we look for previously-uncharacterized genes that mimic the expression patterns of known genes (with or without the types of treatment used in differential expression). The assumption in co-expression is that if the expression of one gene is very similar to the expression of another gene, then it is likely that they are related in their function. Highly similar expression, like highly similar sequence, suggests similar function **[32, 33]**.

Our main aim in the study is to do analysis of the expression pattern of the gene expression microarray data of *Toxoplasma gondii* to find out new probable drug targets with the help of Hierarchical and k-means clustering methods and to do comparison of the results of both methods to confirm the expression pattern. In the next step, the genes co-expressed with the SAG and GRA family members have to identify. Than the promoter sequences of *Toxoplasma gondii* have to be predicted, because there is no prior information about the promoters of *Toxoplasma gondii*. Then sequence alignment has been done to find out the similarity among SAG and GRA family member's promoters and probable target genes promoter's if any.

2. MATERIALS AND METHODS

2.1 Data retrieval and Preprocessing:

Microarray data of *Toxoplasma gondii* was obtained from “The Stanford Microarray Database” (SMD) **[34]**, and all nucleotide sequences were obtained from NCBI (National Centre for Biotechnology Information) **[35]**. After getting microarray data, filtering has to be done and this has been done in the following manner: First, the value of regression correlation was taken to 0.6, this leads to the 4371 genes to pass filters. Then next is deviation filter, this only select genes whose Log (base2) of R/G Normalized Ratio (Mean) is more than 2 standard deviation(s) away from the mean in at least 1 array. This filter removes 3364 genes, leaving 1007 genes. Then only those genes selected who’s Log (base2) of R/G Normalized Ratio (Mean) is absolute value > 2

for at least 1 array(s).This filter removed 431genes, leaving 576.By using genes with > 80% good data ,249 genes were removed and 327 genes were left for further clustering method. Filtering is done using Genesis tool **[36].**

2.2 Clustering:

Hierarchical clustering and k-means clustering were done using Genesis tool. In hierarchical clustering complete linkage clustering method is used. The aim is generally to define clusters that minimize intra-cluster variability while maximizing inter-cluster distances, i.e. finding clusters, in which members are similar to each other, but distant to members of other clusters in terms of gene expression based on the used similarity measurement. Eucledian distance was used to calculate the distance within and among clusters.

2.3 Comparison of expression patterns:

Results obtained by both clustering methods were compared. To validate results clusters were compared to find out similar expression patterns. For a better visibility of the similarity of clusters from different algorithms, a set of clusters is chosen for a comparison.

2.4 Promoter prediction:

Because there is no prior knowledge about the promoters of *Toxoplasma gondii*, promoters have been predicted with Neural Network Promoter Predictor, of BDGP **[37]**. Promoters of the SAG family members were also predicted.

2.5 Promoter analysis:

Promoters of the genes which are present in the same cluster (in which SAG genes are present) are compared by sequence alignment using ClustalW **[38]**.

3. RESULTS AND DISCUSSION

The heat map is generated in the terms of “differential experimental condition”, along with differential expression. Expression image showing 327 genes in sixteen different experiments conducting results from microarray gene expression data of *Toxoplasma gondii*. The color scale ranges from saturated green for log ratios -3.0 and below to saturated red for log ratios 3.0 and above. Each gene is represented by a single row of colored boxes; each column represents an expression value from particular experiment. From these 327 genes, whose expression changed substantially is chosen for cluster analysis using (1) Hierarchical Clustering, (2) k-means. In hierarchical clustering complete linkage clustering method is used. The tree is well distributed and clusters are easily visible by inspecting the ordered expression image. For the *Toxoplasma gondii* dataset, five main clusters marked from one to five and their several sub-clusters were also marked. The clusters have been interactively specified by looking on the patterns in the expression image and then selecting the corresponding sub tree on the left side **(Fig. 1)**.

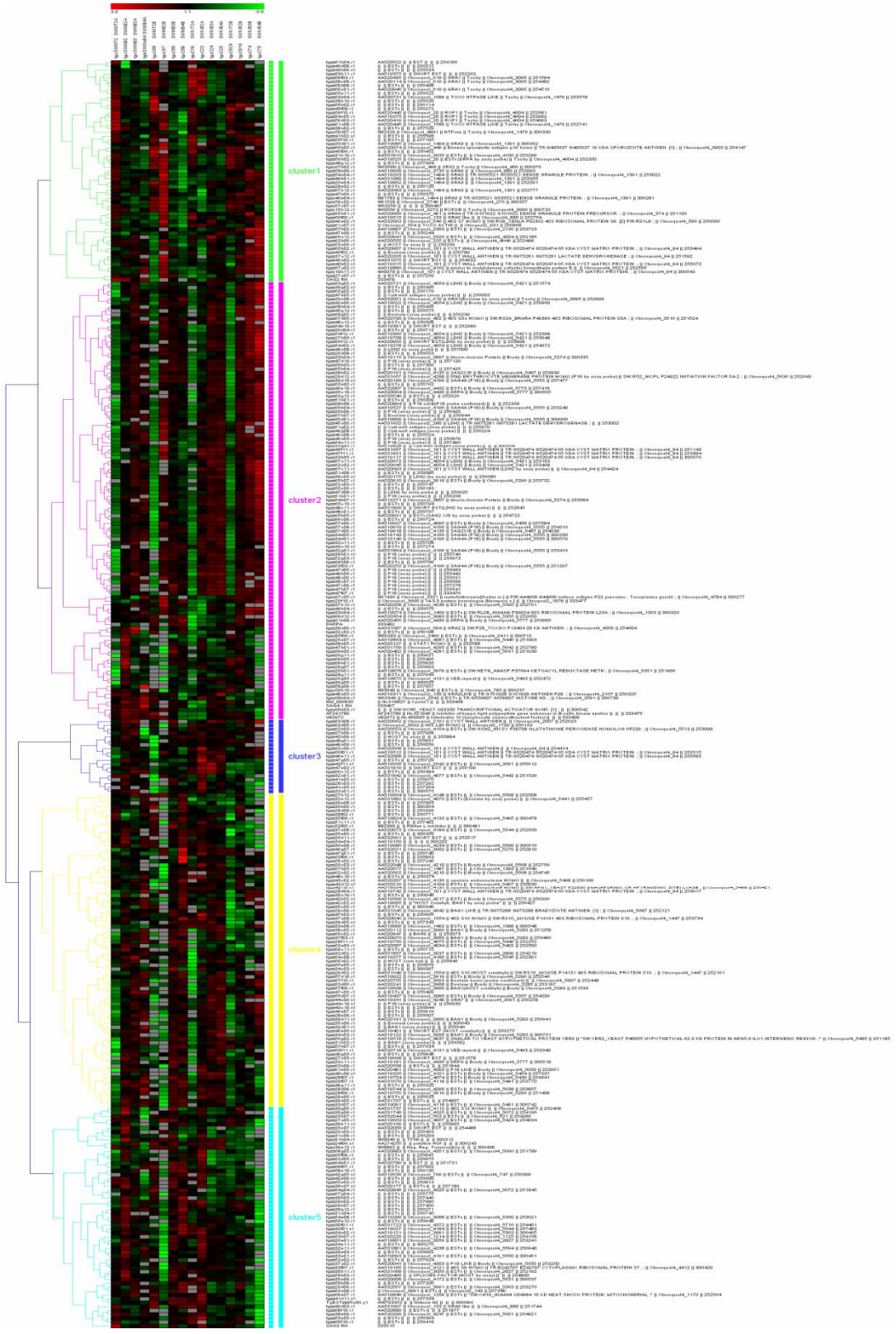

Figure 1: Tree showing Hierarchical clustering using complete linkage. Five main clusters can be clearly seen in the above tree. Each cluster is presented in a different colour to differentiate from other cluster.

By seeing various clusters it can be clearly find out that cluster 2 is the important one for the present work as it has SAG family members **(Fig. 2).**

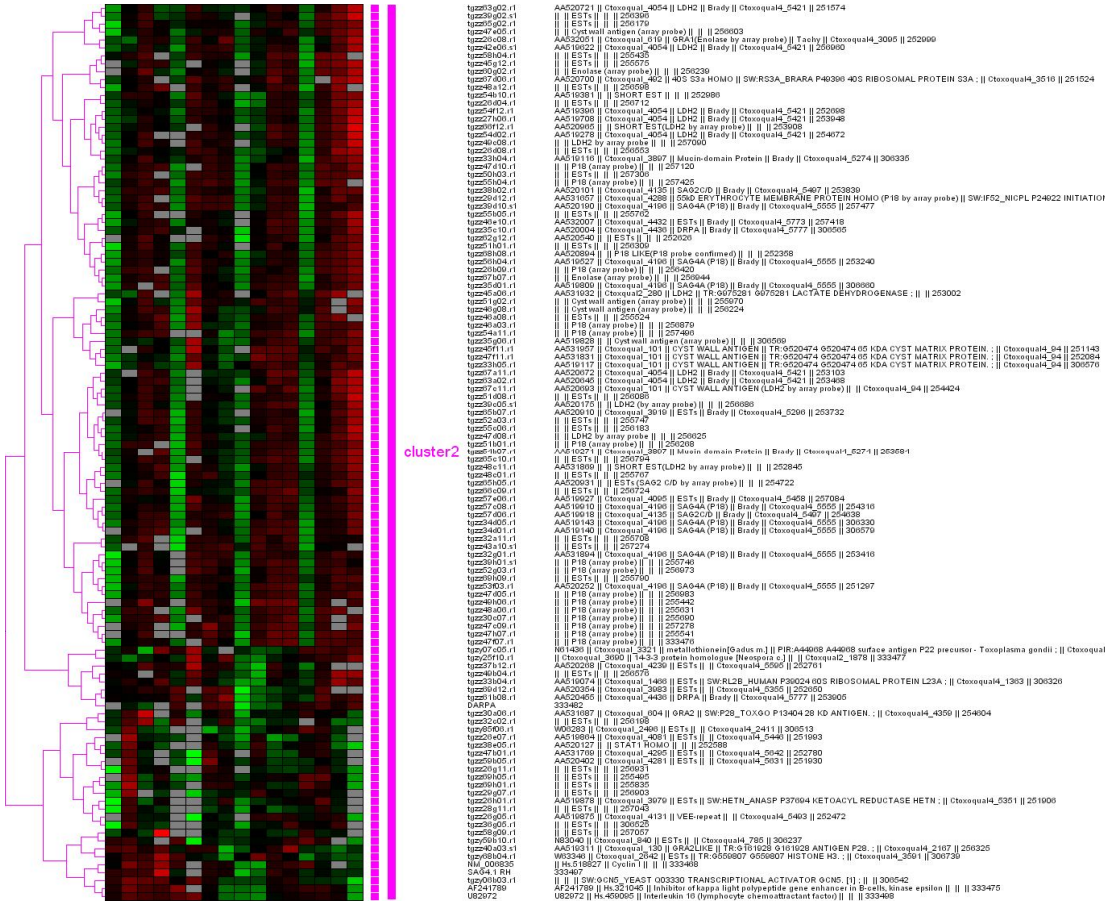

Figure 2 : Expression image of cluster 2. Several subclusters can also be seen in the above image.

In K-means clustering the number of clusters has to be predetermined. Additionally, the maximum number of iteration cycles has to be specified. Usually the algorithm converges (no more reallocations between the clusters) before 50 cycles, but it can occur that the convergence criterion is not reached in a specific time. Therefore, to prevent infinite calculation, the maximum number of cycles has to be declared. For this analysis, k=5 was used for the number of clusters, since 5 basic patterns have been found by hierarchical clustering. The clusters of k-means are very similar to the clusters found with hierarchical clustering. Cluster sizes and gene distribution are of course little different, but both algorithms have found the same basic patterns as the means of the clusters are very similar to each other.

Microarray analysis reveals expression patterns of both previously known and developmentally regulated genes. Stage specific genes e.g. *SRS9,* Ctoxoqual_2199, “mucin domain” protein etc. were clearly identified as such by microarray analysis. Analysis of the microarray data clearly indicates developmental changes in the transcript abundance of *BAG1, SAG4A, LDH2, SAG1* and *NTP1,* which is similar to as published **[19].** Ctoxoqual_819 and Ctoxoqual_4140 were identified as constitutively expressed, as suggested **[19].** Microarray analysis identified several genes whose transcript levels decrease during bradyzoite development. This set includes several previously described genes that were not known to be developmentally regulated: ROP1, ROP2,

ROP4, GRA1, GRA5, GRA8, and MIC1, which is similar to as published **[19].** Constitutively expressed genes include many that encode housekeeping proteins, such as actin and ribosomal proteins. The group of genes that is repressed in bradyzoites includes several that encode proteins that are targeted to the unique secretory organelles of apicomplexan parasites. Of this set, only *NTP*1 has previously been shown to be downregulated during bradyzoite development **[39]**. For the GRA1, GRA5, and ROP1 proteins, previous studies have shown only that these are qualitatively present in bradyzoites. Thus, microarray analysis may be a more sensitive and quantitative method for detecting subtle changes compared to immunofluorescence staining, although changes in transcript and protein abundance will not always parallel each other. The finding that transcript levels of a subset of *GRA* genes decrease during bradyzoite development, while the transcripts for *GRA2, GRA3, GRA4, GRA6,* and *GRA7* are constitutively expressed, shows that selective regulation of *GRA* genes occurs during differentiation, which is similar to as published **[19]**.

The regulated expression of genes encoding rhoptry proteins, if reflected in the protein levels, may explain structural differences in the rhoptries of tachyzoites and bradyzoites. The rhoptries of tachyzoites appear mottled by electron microscopy, while bradyzoite rhoptries are extremely electron dense **[40]**, a phenotype that was also observed in ROP1-knockout tachyzoites. This developmental change could, therefore, be due to decreased expression of ROP1 in bradyzoites. The expression profiles obtained by microarray analysis allowed us to identify distinct classes of temporally regulated genes. Predictions regarding the role of these genes in Toxoplasma development and physiology can be made based on these profiles. A crucial role for the surface proteins SAG2C/D and SAG4A and the unknown protein encoded by the Ctoxoqual_3905 4432 contig is suggested by the early induction and continued expression of these genes at high levels (all-high class), as suggested **[19].**

Microarray data are usually presented as clusters of genes that are grouped based on fold change in transcript levels over time. In such cases, the data reveal changes relative to the starting time or condition, but they do not provide information on whether a given gene's transcripts are abundant or rare relative to other genes. We have employed a technique that determines such relative transcript abundance at each time point, thus allowing distinct expression patterns to emerge from clusters of genes that may otherwise appear to be coordinately regulated using the fold change criteria. Differences in the amount of transcript present for each gene suggest distinct regulatory mechanisms due, for example, to different promoter strengths or different mRNA stabilities. Relative abundance data can also be used to distinguish between "stagespecific" genes (i.e., significant transcript levels in only one stage) and "up- (or down-) regulated" genes (substantial transcript levels in both stages but higher in one than the other).

The identification of distinct classes of regulated genes will make it possible to search for common regulatory sequences **[19]**. For example, the analysis of genes with very high transcript levels and coordinate expression over time may reveal conserved sequence elements in their promoters or untranslated regions, as has been shown for groups of coordinately regulated genes identified by microarray analysis of sporulation in yeast **[41]**. Ongoing *T. gondii* genome sequencing efforts will facilitate such analysis as the sequences needed to identify consensus motifs become available.

To validate clustering results, clusters obtained by both algorithms were compared to find out similar expression patterns, because if different clusters of the above used clustering methods will have similar expression patterns alongwith similar genes then the results will be more useful and reliable. For a better visibility of the similarity of clusters from different algorithms, a set of clusters is chosen for a comparison. Cluster one of hierarchical clustering is similar to cluster five of k-means clustering and cluster two of hierarchical clustering is similar to cluster four of k-means clustering **(Fig. 3 and 4).** Most of the GRA and ROP gene family members are present in the cluster one of hierarchical clustering, are also present in the cluster five of k-means clustering. Similarly, most of the SAG gene family members are present in the cluster two of hierarchical

clustering, are also present in the cluster four of k-means clustering. These results are actually validating our clustering outputs.

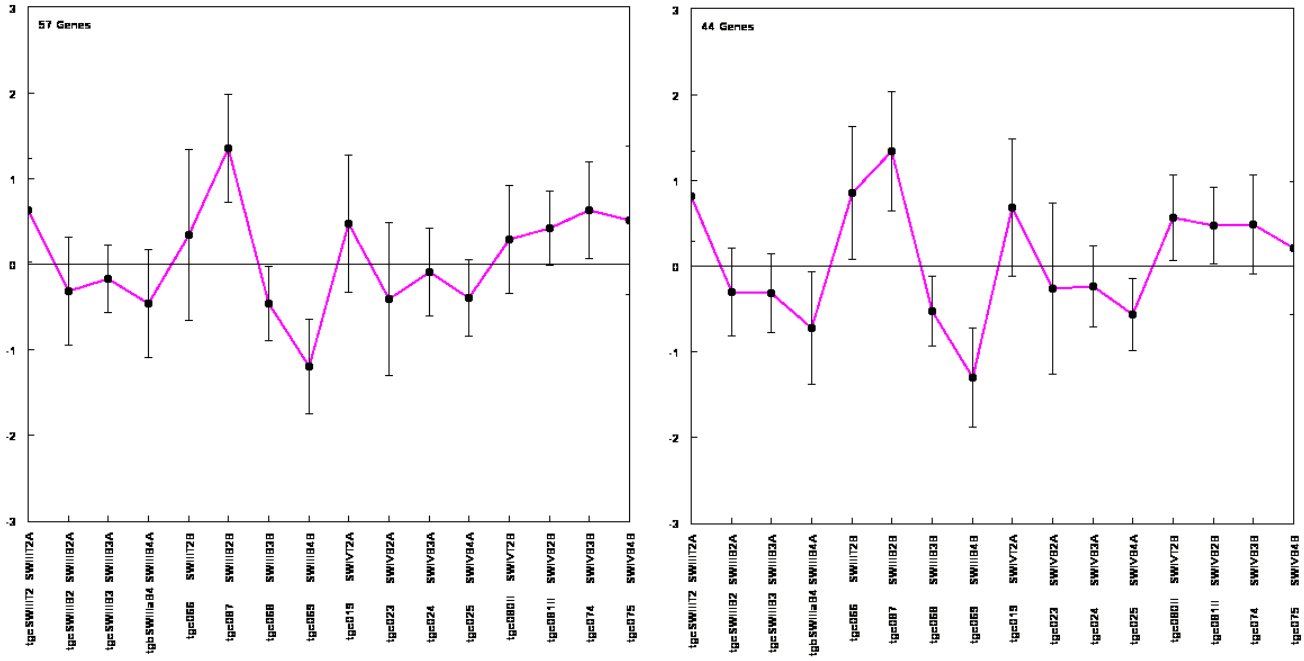

Figure 3 : Expression image of **(A)** cluster 1 of Hierarchical clustering **(B)** cluster 5 of K-means clustering. On the left of the both image expression level is shown into colour ranges from saturated green for log ratio -3 and saturated red for log ratio +3. Both clusters have almost similar expression pattern as both clusters have almost similar average line for expression. Also total numbers of genes are quite similar.

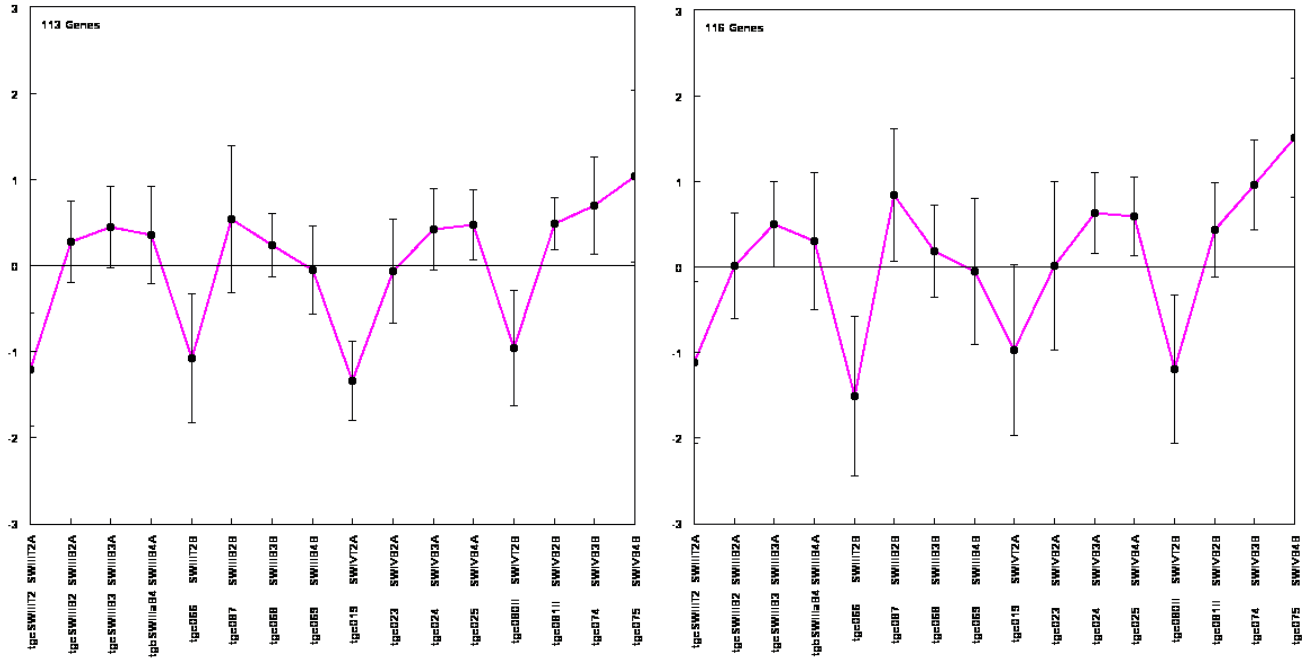

Figure 4 : Expression image of **(A)** cluster 2 of Hierarchical clustering **(B)** cluster 4 of K-means clustering. On the left of the both image expression level is shown into colour ranges from saturated green for log ratio -3 and saturated red for log ratio +3. Both clusters have almost similer expression pattern as both clusters have almost similar average line for expression. Also total numbers of genes are almost similar.

In the promoter analysis, promoters of SAG family and some GRA found in cluster 2 are predicted **(Tab. 1 A)**. Some of the genes in cluster two have been identified as probable targets, these are: U82972 (Interleukin 16), AF241789 (Inhibitor of kappa light chain epsilon), NM_006835cyclin 1(CCNI), W63346 (Histone H3). There promoters are also predicted **(Tab.1 B)**.

Table 1 : Predicted promoters sequences of **(A)** SAG and GRA family members and **(B)** Probable drug targets.

Accession id.	Predicted promoter
(A)	
AA520101 (SAG2C/D)	ACATCTTTTACAAATGTGTCTCACCAGCGTCGGCGCGCGAACAGGTGGGA
AA520190 (SAG4AP18)	GAAGGGGGCAGACCACTGCAACGATGAGCCCGTCGAGCTCGCTGCATTGT
AA519527 (SAG4A(P18))	ATGTGTATCATGCTGCGAACGCATAAAGTACAGTCGAGTGATGCGTGTTA
AA519809 (SAG4A(P18))	TTCTCAAGGTTGAAAAGGGAGACCACTGCAAGGATGAGCCCGTCGAGCTC
AA520931 (SAG2C/D)	GCGAAGAGGAAATAAATGCAGATGTCTTCCACAAAGATGCAACAGCATTG
AA519910 (SAG4A(P18))	TTCTCAAGGTTGAAAAGGGAGACCACTGCAAGGATGAGCCCGTCGAGCTC
AA51991 (SAG2c/D)	ACATCTTTTACAAATGTGTCTCACCAGCGTCGGCGCGCGAGACAGGTGGG
AA519143 (SAG4A(P18))	TTCTCAAGGTTGAAAAGGGAGACCACTGCAAGGATGAGCCCGTCGAGCTC
AA531894 (SAG4A(P18))	ATGTGTATCATGCTGCGAACGCATAAAGTACAGTCGAGTGATGCGTGTTA
AA532051 (GRA1)	CTGCTGTCGCATATGTTTGGGGGGAAATTGCTCGGATATCTTCATTTGGT
AA519311 (GRA2LIKE)	GGCTGCAGATTTGTATAACACAACATGATGTAGCCGCCACGGTTTTTTTT
AA531687 (GRA2)	TGAAGTTCGCTGAAAACGTCGGACAGCACAGTGGGGGGGCGATTATGTTG
(B)	
U82972 (IL-16)	GGGCGAGGGGCTGCACCCACTCTTGTGCCCCAGCAGCCTGAGCAAGTACT
AF241789 (Inhibitor of kappa light chain)	CAGGAGGCCGTGCACAAGCAGACCAGTGTGGCCCCCCGACACCAGGAGTA
NM_006835 (CYCLIN)	CAGCCGTGCGTCCCGCTCGAGCGCCAGCGCCCGCGCCCGCGCCCCCCGAT
W63346 (HISTONE-H3)	TCCATTTTTAAAACGCAAACTCGATTACGCCACCGCTGTCGCACTCTGAC

On comparing these promoters good amount of similarities have been found among these. On analysis of these results we can conclude that there is a good amount of seuqence similarity among the promoters of (1) AA520101_SAG2C/D and U82972_IL-16, AF241789 _Inhibitor of kappa light Chain Epsilon, AA532051_GRA1, AA531687_GRA2, W63346_HISTONE-H3, NM_006835_CYCLIN. (2) AA519809_SAG4A_P18 and U82972_IL-16, AF241789_Inhibitor of kappa light Chain, AA532051_GRA1, W63346_HISTONE-H3, & NM_006835_CYCLIN. But, found low similarity between AA519809_SAG4A_P18 with A531687GRA2. Results are showing similarities in the SAG family with IL-16 and Inhibitor of kappa light Chain Epsilon genes expression pattern. IL-16 gene is an important genes. IL-16 is a multi-functional cytokine that uses CD4 as a receptor to signal diverse biological activities by target cells including T-lymphocytes, monocytes and eosinophils. This cytokine is a functionally significant endogenous antiviral factor. The antiviral activity of IL-16 may be of therapeutic benefit in HIV/AIDS, but its greatest potential is for immune reconstitution. Stimulation of CD4+ T-cells with IL-16 primes cells to respond to IL-2, by up-regulating the expression of IL-2 receptor p75 (CD25). Co-treatment of peripheral blood mononuclear cells (PBMC) with IL-16 plus IL-2 (or IL-15) in vitro selectively expands the population of CD4+ T-cells. In combination with IL-16, the beneficial effects of IL-2 may be augmented and specifically targeted to CD4+ T-cells. Thus, IL-16 shows considerable promise as an agent for the biological therapy of HIV/AIDS and other infectious diseases like toxoplasmosis. Thus IL-16 gene can be a probable drug target in case of the toxoplasmosis. Inhibitor of kappa light Chain Epsilon can also be a probable drug target as it has important functions in immune system. As it is known that, NK and T-cell-derived IFNγ is the critical cytokine in protection against infections with all *Toxoplasma* strains **[42]**. This cytokine protects against *Toxoplasma* infections by upregulating the expression of inducible nitric oxide synthase,

indoleamine dioxygenase, and a family of IFNγ-regulated GTPases that degrade the parasitophorous vacuole **[43]**. Regardless of its effectiveness, some parasites can evade IFNγ-mediated killing and develop into bradyzoites. One possible mechanism by which the parasite avoids IFNγ is to disable IFNγ-induced signaling. Indeed, microarray and cell biological assays demonstrated that IFNγ-induced transcription is abrogated in cells previously infected with *Toxoplasma* **[44, 45]**. In contrast to the polymorphic ROP16 and ROP18 virulence factors, *Toxoplasma*'s effects on IFNγ-dependent transcription are strain independent **[43]**. The mechanism underlying parasite abrogation of IFNγ-stimulated transcription is still unclear but does not appear to involve blocking nuclear localization of STAT1, which is a key IFNγ-regulated transcription factor **[46]**.

4. CONCLUSIONS

Despite enormous development in the technology of gene expression analysis, there are different aspects of the microarray data analyis of the *T. Gondii,* that have been poorly addressed or very less known. Recent evidences strongly improve our understanding about this organism **[46].** Our study proposes some probable drug targets for the treatment of toxoplamosis. As these genes (IL-16 and Inhibitor of kappa light chain) have very important role in immune system in protecting body from any antigenic activities thus, these genes can act as possible drug targets for the treatment of toxoplasmosis. But further study is needed to develop more useful non-resistant drugs for toxoplasmosis.

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