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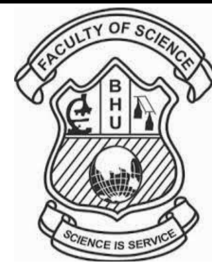

Production and Characterization of a Polyclonal AntibodyAgainst *Tac1p*, Transcription Factor Involved in *Candida albicans*Drug Resistance

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Abstract: **The commensal *Candida albicans* becomes lethal in immunocompromised people. Cases of candidiasis has been drastically increased with emergence of multidrug resistance, not only against the drug in use but also due to collateral resistance against other unrelated drugs.*CDR1* and *CDR2*,ABC transporter genes are upregulated in *C. albicans* developing resistance to azoles. Nuclear localized *TAC1* (transcription activator of CDR genes) was earlier shown to be involved in regulation of *CDR* genes expression in azole resistant (AR) isolates of *Candida albicans*.Gain-of-function mutations in Tac1p has been shown to result in constitutive expression of target gene and developed azole resistance in many clinical *C. albicans* isolates.In the present study, we generated polyclonal antibodies against CaTac1p to understand the basic molecular mechanism of *TAC1* mediated regulation of CDR genes. Half-length ORF of *TAC1* was amplified from SC5314 strain and cloned in pGEX-6P-2 bacterial expression vector. GST-tagged Tac1p recombinant protein was expressed and purified by affinitychromatography method. Polyclonal antisera against Tac1p were raised in mouse model for better understanding on CDR regulation.**

Index Terms:Candida albicans*, *CDR1 **transporter, multidrug resistance, transcriptional regulation, transcription factor.**

I. INTRODUCTION

Candida albicans is an opportunistic human fungal pathogen which causes various infections in human and commensals in gastrointestinal tract with native microflora of human gut. It can cause life threatening systemic infections in immunocompromised patient and vaginal infections in immunocompetent women (Antinori*et al*., 2016; Rad*et al.*, 2016; Sobel, 1994). Such infections are often treated by several antifungal agents for a long time. Azoles are commonly used fungistatic agentsto treat oropharyngeal candidiasis in immunodeficient virus-positive human patients. It has been demonstrated occasionally that repetitive exposure of azoles leads to development of specific drug resistance mechanisms in *C. albicans*.

The most studied mechanism of resistance in *C. albicans* is drug efflux by overexpressing multidrug transporters(Sanglard *et al*., 1995; Sanglard*et al.*, 1997; White, 1997; White*et al.*, 1998).Out of two super families of transporters, the major facilitator superfamily (MFS) having efflux pump MDR1 (multidrug resistance 1) and the ATP-binding cassette (ABC) transporters expressing CDR1 and CDR2 (candida drug resistance), have been shown to be overexpressed in azole antifungal resistance(Prasad*et al.*, 1995; Sanglard *et al*., 1997; White, 1997).Markedly, arbitrary selection of the specific

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substrates and different mechanisms of drug extrusion is used by these multidrug resistant transporters for drug extrusion(Prasad*et al.*, 2006).Transcriptional control of expression of these transporters is an intrinsic process, and studying the underlying mechanisms of regulation by both cis- and trans- acting factors are important for understanding the azole resistance and transport mechanisms in *C. albicans*.

CDR1 and *CDR2* genes are regulated by several elements of *CDR* genes. The regulation of *CDR1*by cis- and trans- acting elements has been documented widely(Chen*et al.*, 2004; Coste *et al*., 2006; Gaur *et al*., 2004; Karnani *et al*., 2004; Puri *et al*., 1999).Until now, a *basal*response element (*BRE*) permitting the basal activity of *CDR1,* and another *drug* responsive element (*DRE*) upregulating the expression of *CDR1* and *CDR2* genesin azole resistant strains have been determined(Micheli*et al.*, 2002). Trans- acting factors *NDt80*and *TAC1*,regulating *CDR1* expression has been identifiedby several researchers(Chen *et al*., 2004; Coste*et al.*, 2004).

In *C. albicans*, Tac1ptranscription factor belonging to the family of zinc-finger proteins with a Zn_2Cys_6 motif binds to *DRE* in both *CDR1* and *CDR2* promoters and transiently activates the*CDR* genes in azole resistant strains(Coste *et al*., 2004). Only Tac1p has been experimentally proved to function as transcriptional activator of transporter genes of *C. albicans*.But the underlying molecular mechanism through which *TAC1* results in transient upregulation of *CDR1* and *CDR2* after drug exposure is not well understood. In the present study, we generated polyclonal antibodies against Tac1p of *C. albicans* clinical isolate to get better insight of *CDR1* regulation at molecular level. Purified antibodies are important for diagnosis and treatment of various disease. In this work, polyclonal antibody has been raised in mice against half-length recombinant protein.

II. MATERIALS AND METHODS

A. *C. albicans strains, culture medium, and culture conditions.*

The wild type laboratory strain SC5314of *C. albicans* was used in this study(Odds*et al.*, 2000).For routine purposes, strain was grown in YEPD (1% yeast extract, 2% peptone, and 2% dextrose) at 30°C with shaking at 200 rpm or in synthetic defined (SD) dropout medium (0.67% yeast nitrogen base without amino acids and 2% glucose)(Lee*et al.*, 1975). A 0.2% aliquot of the amino acid dropout mix omitting the required amino acids was added to SD medium. For the production of recombinant *TAC1,* DH5α, DH10α and BL21(DE3) strains of *E. coli* were used and were maintained in LB medium. Ampicillin (100μg/ml) and kanamycin (50 μg/ml) were supplemented when required.

B. *Isolation of genomic DNA fromCandida albicans.*

Genomic DNA extraction was performed by following method of Heintz and Gong with minor modifications (Heintz & Gong, 2020). Briefly, *C. albicans* cells were grown in YEPD media with moderate agitation overnight at 30° C. Cells were collected by centrifugation at 3000 rpm for 5 mins, resuspended in 1 ml of SOE (1 M sorbitol and 0.1 M EDTA), and pelleted down again. Cells were now resuspended in 0.5 ml SOE, and 20 µl of 10 mg/ml zymolase solution (prepared in SOE) was added to the cells. Cell suspension was incubated at 37° C for 1 hour, with intermittent gentle tumble tossing of the tube after every 15 mins. The cells were collected by centrifugation at 2000 rpm for 1 min, until cells were just pelleted enough. Supernatant was discarded by aspiration. Cells were resuspended in0.5 ml of 50mM Tris, 20 mM EDTA mix by vortexing thoroughly for 1 min. 50 µl of 10% SDS was added to the above suspension, and mixed rapidly by inverting the tube several times. Suspension was incubated at 65° C for 30 minutes. 200 µl of cold 5 M potassium acetate was added and tube was kept in ice for 1 hour. After incubation on ice, cell debris was pelleted down by centrifugation at maximum speed for 5 mins at 4° C. The supernatant was collected in a fresh tube at room temperature. 1 volume of room temperature isopropanol was added to precipitate DNA. Precipitated DNA was recovered by centrifugation at maximum speed for 10 sec, and the pellet was air dried for 10 mins. Dried pellet was resuspended in 150 µl of 1X TE buffer (pH 8.0) containing 10 µg/mL RNase A solution (1.5 µl of 10 mg/ml stock). This mixture was incubated at 30° C for 30 mins. Tubes were chilled on ice and centrifuges at 4° C for 5 mins. Supernatant was transferred to a fresh tube and DNA was again precipitated with 2.5 volumes of ethanol and with added 1/10th volume of 3 M sodium acetate (pH 5.2). The pelleted DNA was washed with 70% ethanol, air dried and finally dissolved in 100 µl of sterile TE buffer (pH 7.4).

C. *PCR amplification ofTAC1.*

The primers amplifying half-length encoding region of*C. albicans TAC1* gene (2946 bp)(ID: 3643755) were deduced from the sequence for GenBank accession number NC 03093 by the using software primer3. Specific primers having *Bam*HI and *Sal*I restriction sites were produced by custom synthesis by sigma Aldrich. Primers used during *TAC1* amplification and cloning: Forward primer 5' CGG GAT CCA TGG ATT TAA AAT TAC CCC CAA CTA ATC C 3' and reverse primer 5' CCC AAG CTT TCA ATT TTT ACG AAA TTC ATT TAA AAC C 3'. PCR (30 cycles) was performed in a total volume of 50 µl containing 10 pM concentrations of each primer, 200 µM conc. of dNTPs, 1.25 units of Pfu enzyme, 5 µl

of 10x rection buffer [20 mM Tris-HCl (pH8.8), 10 mM $(NH4)_2SO_4$, 10 mM KCl, 0.1%(v/v) Triton X-100, 0.1 mg/ml BSA, 2 mM $MgSO_4$ or 20 mM tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% (V/V) Triton X-100 (pH 8.8)] and 50-100 ng conc. of template genomic DNA preparations. PCR conditions used as follows-denaturation at 94° C for 1 min, annealing at 50–55° C for 45 sec, extension at 72° C for appropriate time, followed by final extension for 7 mins.The PCR product was visualized by ethidium bromide staining after electrophoresis on agarose gels (1%).

D. *Gel electrophoresis, digestion, and elution.*

Amplified *TAC1* PCR product was subjected to 1% agarose gel electrophoresis, and band of interest was sliced out of the gel carefully. PCR product was extracted and purified using Qiagen gel elution kit as mentioned by manufacturer. Purified *TAC1* fragment was digested with *Bam*HIand *Sal*I restriction enzymes, and digested DNA fragment was cleared to remove enzymes and extracted and precipitated with ethanol in presence of 0.3 M sodium acetate. Precipitated DNA was washed twice with 70% ethanol and resuspended in TE buffer.

Bacterial expression vector pGEX-6P-2 was also digested with *Bam*HI and *Sal*I for 3 hours at 37°, and treated with CIP (Calf Intestine Phosphatase, NEB) for 1 hour to remove 5' phosphate groups from the linearized digested plasmid DNA. Dephosphorylated plasmid was subjected to agarose gel electrophoresis, and desired DNA fragment was cut out from the gel and purified using qiagen gel elution kit.

E. *Recombinant construct formation.*

The resultant processed *TAC1* fragment was cloned between *Bam*HI and *Sal*I sites of linearized pGEX-6P-2expression vector, by mixing both in 3:1 molar ratio of insert to vector for ligation using Quick ligase ligation kit (NEB). Another rection with only vector without insert was used as negative control (self-ligation). These ligated products were used to transform competent *E. coli* bacterial strain DH5α. Transformed DH5α cells were grown on LB agar media containing ampicillin antibiotic. Various colonies were picked for further screening of the positive clone by double digestion and PCR amplification.Glycerol stocks of the bacterial culture having positive construct were prepared for storage at −80°C.

F. *Expression of C. albicans Tac1p recombinant protein inE. coli.*

*E. coli*strain BL21(DE3) was used for expression and purification of the recombinant protein. BL21(DE3) strain harbouring desired construct pGEX-6P-2/*TAC1* was grown overnight at 37° C in LB medium containing 100 µg/ml ampicillin. 100 ml fresh secondary culture having ampicillin was inoculated with 1 ml overnight grown primary culture (1:100), and allowed to grow until OD 600 reached 0.6-0.8. At this stage, 1 ml aliquot of uninduced cells were removed and saved for gel analysis. Now culture was induced with 1 mM isopropyl β -D-thiogalactoside (IPTG) for 3 hours at 37° C with constant shaking. Induced cells were pelleted down by centrifugation and a small aliquot of these pelleted cells was subjected to SDS-PAGE to check the expression of GST (glutathione S-transferase) tagged-Tac1 protein.

G. *Purification of recombinant GST tagged-Tac1 protein.*

Solubility of the recombinant GST-Tac1 was assessed in native lysate preparation of induced bacterial cells followed by SDA-PAGE. IPTG induced cell pellet was resuspended in cold lysis buffer (50 mM Tris-Cl(pH 7.5), 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, 10 mMbeta-mercaptoethanol, and 1 mM PMSF). Cells were lyzed by lysozyme treatment followed by sonication. Lysate was centrifuged at 10,000 x g for 20 mins at 4° C. Supernatant with soluble fractions was removed in a fresh tube, while pellet was with insoluble fraction of the protein.10 µl of native lysate, supernatant and responded pellet were run on SDS-PAGE to verify the solubility of the recombinant protein. Fusion protein located in pellet fraction in insoluble form was recovered by resuspending the pellet in 0.5% sarcosine containing lysis buffer.The soluble recombinant protein was subjected glutathione Sepharose column with a flow rate of 0.1 ml/min. Column was washed and recombinant protein was eluted with glutathione buffer at 0.3 ml/min flow rate. Different fractions of eluted protein were collected and analysed by SDS PAGE. Fractions containing GST tagged protein were pooled and protein concentrations were determined by the Bradford method using BSA as standard (Bio-Rad, Hercules, CA).

H. *Production of polyclonal antibodies.*

Purified recombinant GST-tagged Tac1 protein was used as an immunogen. Swiss albino mice were immunized by subcutaneous injection with around 200 µg of the purified protein, which had been emulsified in Freund's complete adjuvant in 1:1 ratio (v/v)for micelle formation. This was followed by two booster doses of 100 µg protein in incomplete Freund's adjuvant at 3 weeks interval. After 2 weeks, blood sample was collected and the serum was obtained by centrifugation of the clotted blood and stored at -20° C.

L. *SDS PAGE and Immunoblotting.*

SDS PAGE was performed purified recombinant Tac1 protein according to Laemmli's method (Laemmli, 1970). A wide range of molecular weight marker was used to identify and analyse the molecular weight of separated proteins observed in the Coomassie brilliant blue stained gel.To further characterize the specificity of the produced polyclonal antibodies, *C. albicans* lysate preparation was separated by SDS PAGE and electrophoretically transferred to polyvinylidene difluoride membrane (PVDF) (Sigma). Immunochemical staining was performed by blocking the membrane with Tris-buffered saline (50 mM Trish-HCl, 150mM NaCl [pH 7.5]) containing 5% bloto (Genotech). Mice antisera was used to probe the transferred proteins on the membrane. After washing step, bound antibodies were detected by HRP conjugated secondary antibodies (1:10,000 dilution), and detected with enhanced chemiluminescence ECL method according to the instructions of the manufacturer (Biorad).

III. RESULTS

A. *Cloning and expression of TAC1 gene*

To investigate the role of *TAC1* in *CDR1* transcription regulation in *Candida albicans* drug resistance, amplification of half-length orf19.3188of *TAC1*resulted in a PCR product of ~1.2 kb (Fig. 1a), and the PCR productwas cloned in bacterial expression pGEX-6P-2 vector between *Bam*HI and *Sal*I restriction sites (Fig. 1b). After transformation, in negative control agar plate, no colony of bacteria was observed. From the transformed plates, positive clones were picked, screened and confirmed by colony PCR and restriction digestion. A similar sized specific product was obtained by *Bam*HI-*Sal*I double digestion of pGEX-6P-2/*CaTAC1* (Fig. 1c). The result concluded the proper cloning of *CaTAC1* into pGEX-6P-2, supported by sequencing.

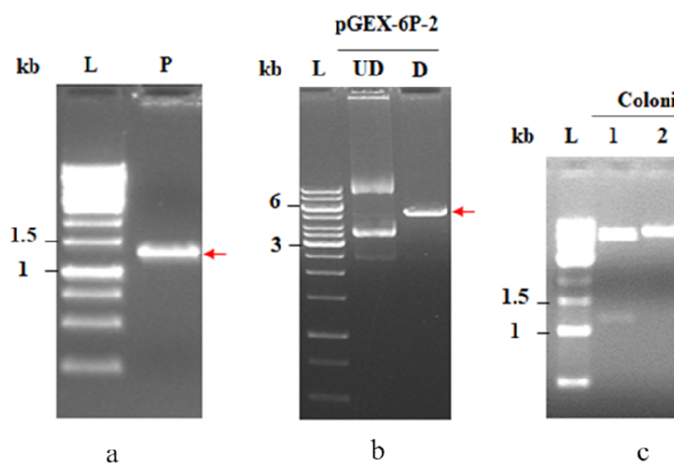

Fig. 1. Construction and confirmation of recombinant *TAC1* of *C. albicans*.(a) PCR amplicon of half-length *CaTAC1* (~ 1.2 kb). kb: kilobase, L: ladder, P: PCR product of *CaTAC1*. (b) Restriction digestion of pGEX-6P-2 (~5.4 kb)with restriction enzymes *Bam*HI and *Sal*I. UD: undigested plasmid, D: digested plasmid. (c) Confirmation of the clone by digesting various colonies with restriction enzymes *Bam*HI and *Sal*I. Only colony 1 shows band of insert.

After confirmation of the recombinant *CaTAC1* clones, the expression conditions of GST tagged Tac1 in bacterial expression system were optimized by inducing the expression with 1 mM IPTG. After standardizing all optimal conditions of temperature and time for induction, an increase in protein expression is observed (Fig.2).The expressed protein identified by SDS-PAGE produced a specific band of ~72 kDa which corresponds to the predicted molecular weight of GST tagged recombinant protein.

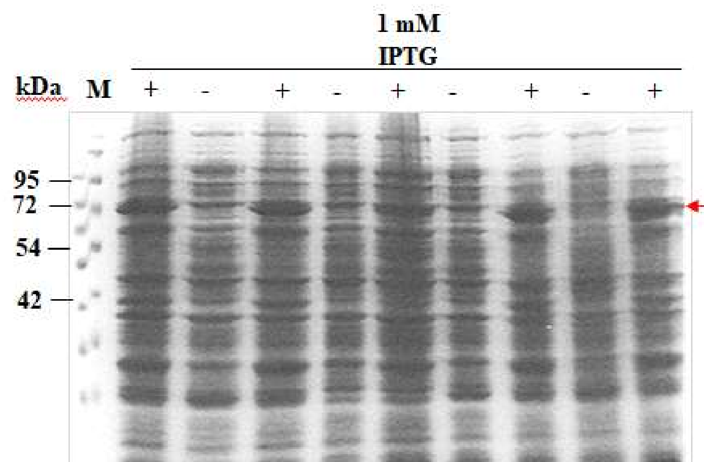

Fig. 2. Induced expression profile of recombinant GST tagged Tac1p.Recombinant protein was induced with 1 mM IPTG at 37° C for 3 hours with constant shaking, and the expression was checked by staining the SDS-PAGE gel with Coomassie brilliant blue. M: marker, +: presence of IPTG (Induced sample), -: absence of IPTG (Uninduced). A specific band of GST-Tac1p at ~72 kDa is indicated by an arrow.

B. *Purification of GST tagged Tac1p recombinant protein*

In order to purify the recombinant protein, induced recombinant protein expressing cells were lysed via sonication and the solubility of the desired protein was checked by SDS-PAGE. We observed the expression of GST tagged Tac1 in insoluble fraction (pellet)(Figure 3a), so it wasnot possible to purify it directly. To resolve it, insoluble fraction was resuspended in 0.5% sarcosine detergent containing lysis buffer. Solubilized GST tagged fusion protein was then purified using glutathione Sepharose matrix. Bound

protein was eluted with reduced glutathione added to the elution buffer. Profile of eluted recombinant protein was checked by SDS-PAGE and bands of purified GST tagged Tac1 protein were observed (Figure 3b).

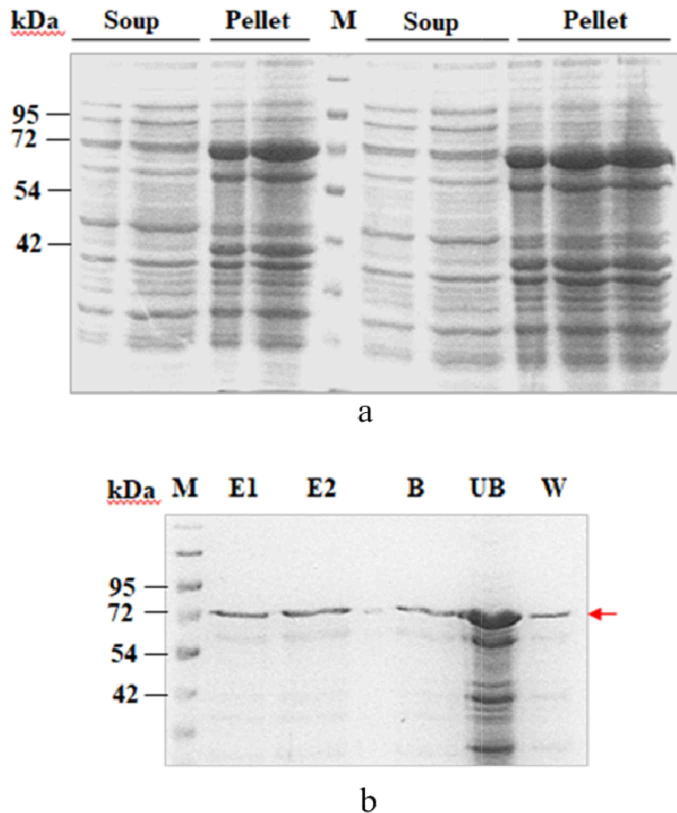

Fig. 3. Solubility and purification analysis of recombinant GST tagged Tac1p.(a) 10% SDS-PAGE shows the expression of recombinant Tac1p in insoluble fractions (pellet), until the 0.5% sarcosine was added to the lysis buffer to get the protein in soluble fractions (soup). (b) Recombinant GST tagged Tac1p purified by affinity purification. M: marker, E1: elution 1, E2: elution 2, B: bead bound fraction, UB: unbound fraction, W: wash out.

C. *Production of polyclonal antibody against recombinant CaTac1p*

Polyclonal antibodies were raised against GST tagged protein in mice as describes in materials and methods. The specificity of the anti-*Ca*Tac1 antibody in pre-immune and immune serum was confirmed by using total cell extract of *Candida albicans* cells (Figure 4). We could observe single specific band in western blot analysis corresponding to Tac1 protein, thus no cross reactivity was observed with *Candida* proteins. We used anti Tac1p antibody at 1:10,000 dilution for further studies.

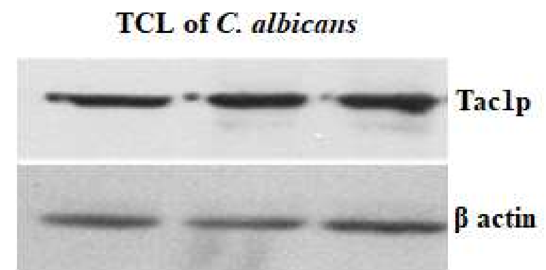

Fig. 4. Confirmation of anti *Ca*Tac1 antibody specificity. Western blot analysis of *C. albicans* total cell extract (TCL) with mice produced polyclonal antibody at 1:10,000 dilution. Bands shows specificity of produced antibodies that could recognize endogenous Tac1p. β actin was used as endogenous loading control.

IV. DISCUSSION

Candida albicans exist in microbiome of immunocompromised as well as healthy individuals (Ford *et al*., 2015). *Candida albicans* is the most frequent species among the current fungal pathogens, followed by other *Candida* species (*Candida glabrata, Candida parapsilosis, Candida tropicais*), non-*Candida* species (*Aspergillus fumigatus*, *Microsporumcanis*) (Lass-Flörl, 2009).These fungi are responsible for various forms of diseases, ranging from superficial infections to systemic infections. Continuous use of antifungal chemotherapy causes acquisition of drug resistance. Various mechanism contributes to acquire drug resistance in *Candida abicans* like transport alterations, target alteration by mutation and gene upregulation, utilization of compensatory and catabolic pathways and over expression of efflux pumps(Sanglard*et al.*, 2009). Major mechanism involved in drug resistance is over expression of efflux pumps(Prasad & Rawal, 2014).Azole antifungal resistance in clinical *C. albicans* isolates often is triggered by the upregulation of genes encoding multidrug efflux pumps i.e., ABC transporter *CDR1* and *CDR2*. The transcriptional regulation of CDR1 is an intricate system which includes its transient induced expression by triggering various stresses and activation by several transcription activators(Chen *et al*., 2004; Coste *et al*., 2004; Krishnamurthy*et al.*, 1998; Murad *et al*., 2001).*CDR1* overexpression in azole susceptible (AS) and azole resistant (AR) isolates has been shown due to an enhanced transcriptional activation and increased mRNA stability(Gaur *et al*., 2004; Manoharlal*et al.*, 2008).*CDR1* is prominently regulated transcriptionallybyTac1p as previously reported (Coste *et al*., 2006). Tac1pis often associated with gain-of-function mutations resulting in hyper resistance in clinical*Candida*isolates.In this study, we attempt to characterize the role of Tac1p to identify its

functional role in *Candida albicans* drug resistance mechanism.

In *C. albicans* clinical isolates resistant to azoles, deletion of *TAC1* abolished *CDR1/CDR2* expression and therefore drug resistance, thus demonstrating that *TAC1* is a major mediator of azole resistance due to the upregulation of the ABC transporter(Coste *et al*., 2004). *TAC1*acts by direct binding to the *DRE*which is present in the promoter region of both efflux pump genes and induce their expression in response to steroid and several toxic chemicals(Coste *et al*., 2004; De Micheli *et al*., 2002; Liu*et al.*, 2018).Remarkably, the binding of *TAC1* to the promoters of *CDR1* and *CDR2* have also been validated bychromatin immunoprecipitation (ChIP)-on-chip assays (Liu *et al*., 2007).

Thus,the characterization of Tac1pwould be helpful in the identifying the role of this protein in basal transcription, pathogenesis and drug resistance mechanism. To characterize the role of *TAC1,* we first generated anti-Tac1ppolyclonal antibody. The advantage of generation polyclonal antibody is that it is accessible to any part of protein and increased sensitivity of detection due to availability of more epitopes.Polyclonal antibodies have various therapeutic role, and polyclonal antisera is mainly used in replacement therapies where patients suffering from immune deficiency disease or neutralization of toxins and viruses. Availability of specific antibody help us to validate the function and fundamental process of particular protein (Arur & Schedl, 2014).

CONCLUSION

In the present study, we successfully purifiedTac1pprotein. The antibody generated have high specificity which was confirmed by western blot analysis. The titre value of antibody was 1:10,000 which is quite high. Through this study, we were able to generate highly specific antibody against Tac1p. Similar type of work was done byKlekamp and Weil, where they generate antibody against *Saccharomyces cerevisiae* transcription factor TFIIIB(Klekamp & Weil, 1986).These antibodies used to analyse phase specific expression. We checked differential expression of Tac1pin different phases of growth. These antibodies will be helpful to perform Co-IP experiments which will help us to get better insight of the mechanism through which *TAC1* regulates *CDR1* expression and Co-IP analysis also revealed interacting partners of Tac1p. Further studies are necessary to understand the exact role of *TAC1* in *Candida albicans* drug resistance mechanism.

CONFLICT OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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