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Research Article

MOLECULAR CLONING OF ADH GENE OF *CANDIDA ALBICANS* AND EVALUATION OF ITS IMMUNOGENICITY *IN VITRO* AND *IN VIVO*

Aditi Grover¹, B.S. Bhandari¹, Nishant Rai^{2*}

¹Department of Microbiology, H.N.B. Garhwal University Srinagar, Pauri Garhwal, 246174, Uttarakhand, India.

²Department of Biotechnology, Graphic Era University, Dehradun -248001 (Uttarakhand), India. *Corresponding Author: nishantrai1@gmail.com

ABSTRACT

The *Candida albicans* genomic DNA was isolated and confirmed on Agarose gel (0.5%). ADH gene (1305bp) was amplified using specific forward (31mer) and backward primers (29mer). This amplified ADH gene was ligated in pTarget expression vector (5.67kb) and transformed in *E.coli* DH5 α cells. The gene insert was confirmed by RE digestion, colony PCR and gene sequencing. Expression of recombinant plasmid DNA, pTarget.caadh was done in HeLa cells. Transfected HeLa cell monolayer were detected by immunoperoxidase test and positive staining was observed showing expression of ADH gene product. Immunogenic study in mice was performed by immunizing four-week-old Swiss albino mice with pTarget.caadh DNA 50 μ g in 0.5ml per mouse intramuscularly. The antibody in sera of mice was assayed by ELISA and found to be positive as compared to healthy control group of mice. It was found that ADH gene is immunogenic as evidenced by high antibody titre (512 and 1024 after 21 and 28 days respectively). The immunized mice were challenged by *Candida albicans* (4×10^4 CFU in 200 μ l of sterile PBS) after 28 days of immunization and percent protection was found to be 72%. Present findings showed that ADH is an ideal immunogen and could be a potent candidate for anticandida vaccine preparation.

Key words: *Candida albicans*, ADH gene, anticandida vaccine, immunogenicity, Percent protection.

Isolation of genomic DNA was done following the protocol of Harju *et al.* (2004). The isolated DNA was confirmed by performing 0.5% agarose gel electrophoresis. The PCR was carried out following the protocol of Sambrook and Russell (Sambrook and Russell, 2001). The *Candida* genomic DNA (ADH gene) was subjected to PCR amplification in 50 μ l reaction mixture with 10x Taq DNA buffer (5 μ l), dNTPs mix (10mM each, 4 μ l), primer F and primer R (1 μ l each), template DNA (6 μ l), nuclease free water (30 μ l), Taq DNA polymerase (2.5U, 1 μ l) and 5mM MgCl₂ (3 μ l). The specific F and R primers were used in the concentration 50 pM. The PCR set up was performed in Thermalcycler (Quanta Biotech, UK) with cycling conditions programmed with a temperature profile: 95°C for 4 mins (1 cycle); 94°C for 1 min, 56°C for 1 min and 72°C for 1 min (35 cycles); 72°C for 10 mins (1 cycle). The amplified gene product was then confirmed by 1% and 0.7% agarose gel electrophoresis.

Ligation Reaction

The ligation reaction was carried as described by Rai *et al.* (2010). The amplified ADH gene (2 μ l) was ligated to pTargeT expression vector (1 μ l) with T4 DNA ligase (1 μ l) in 10X ligation buffer (1 μ l) in a total volume of 10 μ l. The ligation reaction mixture was centrifuged briefly, kept on ice and was incubated at 4°C overnight.

Competent Cell Preparation and Transformation

One step competent cell preparation and transformation were done using the methodology of Chung *et al.* (1989). Transformants were selected by standard

methods. Individual labeled *E. coli* DH5 α cells with white morphology were selected for amplification in 5ml LB broth containing ampicillin (100 μ g/ml) and put in orbital shaking incubator (Remi) at 37°C at 200 rpm for overnight growth. These bacterial cultures were subjected to plasmid DNA isolation.

Isolation of Plasmid DNA by TELT Method

The plasmid DNA isolation was performed according to the method of Ausubel *et al.*(1990).

Identification of Positive Clones

Gene in right orientation was identified by RE analysis, amplification of ADH gene and sequencing of insert using T7 primer (Sambrook and Russell, 2001). The caadh abbreviation was given for *Candida albicans* ADH gene. The digestion reaction of recombinant plasmid DNA (pTargeT.Caadh) was carried out in 0.5 ml microfuge tube containing 1X reaction buffer (1 μ l), *Pst*I (1 μ l), target DNA pTargeT.Caadh (5 μ l) and nuclease free water (3 μ l) in a total volume of 10 μ l. The reaction mixture was mixed and was spun at 5000 rpm for 15 sec. It was incubated at 37°C for 2h in water bath and was checked on 1% agarose gel.

In colony PCR, T7 was used as forward primer and ADH gene specific R primer as reverse primer.

The recombinant plasmid DNA was subjected to PCR amplification in 50 μ l reaction mixture with 10X Taq DNA buffer (5 μ l), dNTPs mix (10mM each, 4 μ l), primer F and primer R (1 μ l each), template DNA (6 μ l), nuclease free water (30 μ l), Taq DNA polymerase (2.5U, 1 μ l) and 5mM MgCl₂

(3µl). The specific F and R primers were used in the concentration 50 pM. The PCR set up was performed in Thermalcycler (Quanta Biotech, UK) with cycling conditions programmed with a temperature profile: 95°C for 4 mins (1 cycle); 94°C for 1 min, 56°C for 1 min and 72°C for 1 min (35 cycles); 72°C for 5 to 10 mins (1 cycle). The amplified gene product was then confirmed by 1% and 0.7% agarose gel electrophoresis. The ADH gene in the recombinant plasmid was sequenced using T7 primer to confirm its right orientation.

Expression studies

The transfection reaction was done as described by Rai *et al.* (2010). Growing HeLa cells were harvested exponentially by trypsinization and cell suspension was prepared using growth medium. The calcium phosphate DNA co-precipitate was prepared by combining 100µl of 2.5 M CaCl₂ with 20µl of plasmid DNA in a sterile microfuge tube. 80µl nuclease free water was added and kept at RT for 1 minute. Immediately the calcium phosphate DNA suspension was transferred using 20µl suspension to each well of the microplate. 100µl of cell culture suspension was added in each well. The plate was rocked gently to mix the medium which became yellow orange and turbid. The DNA precipitate was formed and kept control wells without transfection. The plates were incubated at 37°C in a humidified incubator with an atmosphere of 5% CO₂ for 72 hours.

Immunoperoxidase Test (IPT) for Detection of Expressed ADH Glycoprotein in Cell Culture

The procedure described by Rai *et al.* (2010) was followed. The medium from

wells of culture plate was removed. The transfected and control wells were processed. The monolayer was washed with PBS (pH 7.2). The cells were fixed with chilled acetone at 4°C for 10 min and then air-dried. Then few drops of mouse anticandida ADH serum was added and incubated at 37°C for 1h in a humid chamber. Also one well was marked as control. Then the wells were washed with PBS. Few drops of the goat antimouse IgG-HRP conjugate were added and incubated at 37°C for 1 h in a humid chamber. The wells were washed three times with PBS then it was air-dried. Three drops of freshly prepared Nadi reagent was added on the preparation and allowed to react for 2min. The preparation was rinsed briefly with PBS and treated for about 1 min with each of the following: 70%, 90% and 100% alcohol followed by a quick wash with xylene. The monolayers were observed under inverted microscope.

Immunogenicity Studies in Mice

Four-week-old Swiss albino mice were immunized with pTarget.caadh DNA (50µg in 0.5ml per mouse) intramuscularly into quadriceps (Gastrocnemius muscle) two times at seven days interval using disposable syringe. In control mice groups, pTarget vector DNA was injected and healthy controls were kept separately.

ELISA

It was performed as described by Rai *et al.* (2010). *Candida albicans* cells were diluted with coating buffer and added 50µl to each well of ELISA plate. The plate was sealed and incubated overnight at 4°C. The solution was decanted from wells, plates were washed thrice with PBST and shaken

between each washing. 200µl of blocking solution was added to each well. The plate was sealed and incubated at 37°C for 1-2 hours. The blocking solution was decanted and the plate was again washed thrice with PBST. Thereafter, 50µl of the mouse sera sample was added per well and incubated at 37°C for 2 hours. The unbound serum was decanted and wells were washed thrice with PBST. 50µl of the goat anti-mouse-HRPO conjugate at 1:100 dilutions was added to each well and the plate was incubated at 37°C for 2 hours. The unbound conjugate was removed from the wells and the wells were washed thrice with PBST. 100µl of the substrate solution was added to each well and incubated at room temperature for 10 minutes. The reaction was stopped by adding 50µl of 5N sulphuric acid to each well. The color developed, was observed by identifying positive and negative samples and absorbance was taken at 450 nm.

Percent Protection in Mice

For immunization, mice were inoculated with pTarget.caadh DNA alone having dose of 50µg in 0.5ml per mouse. Mice were challenged after 28 days of immunization with *C. albicans* (SC5314 strain). The cells (4×10^4 CFU) were diluted in 200µl of sterile PBS and injected via tail vein to induce disseminated candidiasis. Four groups were made having 25 mice in each group viz., group A (Immunized with pTarget.caadh and challenged), group B (Immunized with pTarget vector DNA and challenged), group C (Not immunized and challenged) and group D (Not immunized and not challenged). These were kept under

observation for 20 days and percent protection was calculated using the formula:

$$\frac{\text{No. of live mice left}}{\text{Total no. of mice taken}} \times 100$$

RESULTS

Cloning of *Candida albicans* ADH Gene

Genomic DNA was isolated from *C. albicans* and an ADH gene was amplified using specific forward and reverse primers. The amplified gene was then ligated in pTargetT vector and transformed in competent cells.

Overnight grown recombinant culture in LB broth having ampicillin (100µg/ml) showed visible growth as the culture became turbid. Electrophoresis of isolated genomic DNA showed band at 57.5 kb level. A clear band at 1.305 kb on 0.7% agarose gel confirmed the amplification of the PCR product (Fig. 2).

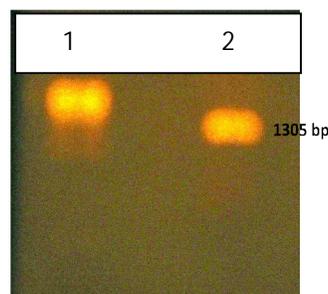


Fig. 2. 0.7% Agarose gel electrophoresis of amplified ADH gene of *Candida albicans*. Lane 1- 1 kb DNA Ladder (Fermentas); Lane 2- ADH Gene of 1305 bp.

Amplified product of ADH gene (1.305 Kb) was ligated to pTargetT expression vector and then transformed into competent *E. coli* (DH5α) cells. Positive clones were observed (white colored

colonies). Twelve white colonies were randomly selected and plasmid DNA was isolated from 4 clones numbered 1, 2, 3 and 4 to identify right orientation of ligated gene.

Overnight grown *E. coli* (DH5 α) culture containing recombinant plasmid in LB broth having ampicillin (100 μ g/ml) showed visible growth as the culture became turbid. pTarget.Caadh DNA was isolated from culture using TELT method and a clear band of 6.975 kb (as expected) was seen on 0.7% agarose gel. The yield was 2-3 μ g pure DNA per 1.5ml culture.

Digestion of pTarget.Caadh with *Pst*I along with its specific assay buffer produced three fragments of 3.658 kb, 2.821 kb and 0.496 kb size on 0.7% agarose gel that confirmed the right orientation of ADH gene. Same results were also analysed by DNA Star software.

The pTarget.Caadh DNA containing ADH gene was amplified using T7 (Forward primer) and ADH gene specific R primer (reverse primer). A clear band at 1.305 Kb was seen on 0.7% agarose gel.

By sequencing of ADH gene in the recombinant plasmid using T7 primer its right orientation was confirmed.

Expression studies

Recombinant plasmid DNA (pTarget.Caadh) transfected in HeLa cell monolayers were analysed by IPT for expression of ADH protein in cell culture. Large number of cells in the monolayer revealing positive staining under inverted microscope were observed showing expression of ADH protein. Control wells showed no color development. The HeLa cells that were transfected with

pTarget.Caadh were also analyzed by ELISA to check the expression of ADH gene. The ADH antigen was detected in the transfected cells while control cells showed no antigen as observed by color development (Fig. 3, 4).

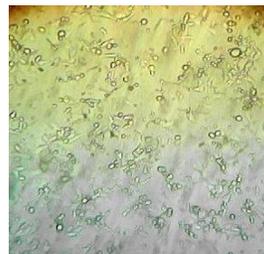


Fig 5. Mock transfected HeLa cells showing no color in IPT.

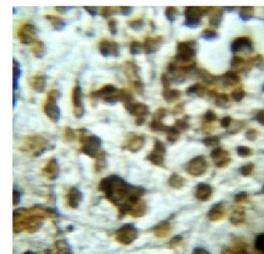


Fig 6. HeLa cells transfected with pTarget.Caadh showing brown color development in IPT.

Immunogenicity Studies in Mice

pTarget.Caadh vaccinated mice had antibody titre of 512 and 1024 after 21 and 28 days respectively whereas the pTarget vector group alone showed an equal amount of antibody titre i.e. 8 after 21 and 28 days. Healthy control group showed no antibody titre value.

The vaccinated mice were challenged with *Candida albicans* (4×10^4 CFU) after 28 days of immunization. Upto 5 days after challenge, death of mice was considered as non-specific death. The 6th day was taken as base line and the mice were observed upto 20 days. In group A (immunized with pTarget.Caadh and challenged) the number of mice protected were 18 out of 25 showing that the pTarget.Caadh group gave moderate percent protection of 72. Whereas, zero percent protection was observed in the mice of group C (not immunized and challenged) with sudden paralytic symptoms, dullness and unconsciousness resulting into their

deaths. In group B (immunized with pTarget vector DNA and challenged) only 01 mice was protected out of 25 showing only 4% protection. In group D (not immunized and not challenged) 24 mice were live.

DISCUSSION

With a view to develop a suitable DNA vaccine, cloning, expression and immunogenic study of ADH gene were done.

In the present work, genomic DNA of *Candida albicans* was isolated and its size (57.5kb) was confirmed by 0.5% agarose gel electrophoresis. Kurtz and Loeffler (Kurtz *et al.* 1986; Loeffler 2002) have also reported the isolation of genomic DNA.

The sequences of ADH gene of *Candida albicans* were downloaded from the database of GenBank/NCBI. The optimal annealing temperature obtained at primer designing was 56.6°C with a product length of 1305bp. So the annealing temperature of PCR was kept at 56°C. The sequences were aligned using Megalign and Seqman programme of DNA Star software. This amplified PCR product as clear band was confirmed on 0.7% agarose gel. In a similar study by Park (Park *et al.* 2006) two PCR primers were designed on the basis of amino acid sequences of *C. utilis* ADH1.

This amplified gene could be easily ligated in pTarget vector and the recombinant vector (pTarget.Caadh) was transformed in *E. coli* (DH5 α) competent cells.

The recombinant plasmid pTarget.Caadh DNA containing *Candida*

albicans ADH gene (1.305 kb) was also confirmed by amplification using specific forward and reverse primers. Sequencing of ADH gene in the recombinant plasmid was done using T7 primer and its right orientation was confirmed. In a similar study by Marina (Marina, 2011) pYes2 vector was used as a cloning vector for ligation of ADH1 gene and the orientation of insert was checked by digestion with *Kpn*I.

Recombinant DNA containing the insert in right orientation was then transfected in HeLa cells to check the expression of the ADH protein to be immunogenic. Assay of expression of pTarget.Caadh plasmid DNA was confirmed by immunoperoxidase test as described by Rai *et al.*(2010). In this test, the transfected cells showed color change with chemicals and visible under microscope because these cells were expressing proteins but healthy control did not show any color change. The present study revealed that the plasmid DNA encoding ADH gene is expressing the ADH gene product in fully functional form in mammalian cells. In a similar approach (Wang *et al.* 2006; Xingmin *et al.* 2006) expression of the cloned protein (CaADH1p) in pYES2 plasmid was confirmed by Western blot using anti-His antibody.

Molecular cloning and characterization of the alcohol dehydrogenase ADH1 gene of *Candida utilis* ATCC 9950 was also done by Park *et al.* (2006). The sequence analysis and enzyme characterization of *C. utilis* ADH1 suggested that *C. utilis* ADH1 might be a primary alcohol dehydrogenase present in

the cytoplasm and requiring zinc ion and NAD(P)⁺.

The present study showed that ADH gene is immunogenic as evidenced by high antibody titre (512 and 1024 after 21 and 28 days respectively). The antibody titre was detected by ELISA technique that was in agreement with Bromuro *et al.* (1998). The high antibody titre may protect the mice against antigens (Mencacci *et al.* 1994; Han and Cutler, 1995) in which the protection in mice immunized by definite antigens and in stringent models of lethal infection by *C. albicans* was obtained. The mice vaccinated with recombinant plasmid DNA (pTargeT.Caadh) did not show any adverse effects evidencing that the vaccine is safe.

Present study showed a significant finding that mice showed immune response after DNA vaccine administration. Challenge of mice through tail vein with *Candida albicans* showed that pTargeT.Caadh DNA used for vaccination gave 72% protection which was significantly higher than vector DNA alone (20%) and healthy control groups (0%).

The immunization in mice revealed that gene is capable of inducing production of ADH specific antibodies, thus it can be a suitable alternative to the currently used *Candida* vaccine. Further studies are needed to augment the efficacy of the vaccine by using chemical adjuvants, liposomal encapsulation and conjugation with carrier protein (Maecker *et al.* 1995; Barouch and Letvin, 2000; Jin *et al.* 2004; Chen *et al.* 2009). There seems to be no earlier report revealing the efficacy of ADH gene as DNA vaccine against *Candida albicans*. Multivalent vaccine approach against

Candida albicans is suggested recently by Cassone (Cassone, 2013).

The above mentioned studies continue to build upon the fundamental knowledge of protective immunity against *Candida*, which should ultimately lead to development of a clinically useful vaccine.

CONCLUSION

It was found that ADH gene from *C.albicans* is immunogenic as evidenced by high antibody titre (512 and 1024 after 21 and 28 days respectively). The immunized mice challenged by *Candida albicans* (4x10⁴ CFU dissolved in 200µl of sterile PBS) showed 72% percent protection. Present findings showed that ADH is an ideal immunogen and could be a potent candidate for anticandida vaccine preparation.

The present work describes a systematic investigation of the protective ability of *C. albicans* ADH gene in candidiasis. Present findings showed that ADH gene expressing is an ideal immunogen and could be a potent candidate for anticandida vaccine preparation.

Conflict of Interest (COI):

The authors declare that they have no conflict of interest in the publication.

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