Expression of 3AB protein of foot and mouth disease virus in Pichia pastoris

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3AB non structural protein (nsp) was used to diagnose the vaccinated animals from those infected with foot and mouth disease virus (FMDV). In order to express the gene encoding 3AB protein of FMDV type A22 in Pichia pastoris, the gene was amplified and cloned into the yeast transfer vector (pPIC-9K) at EcoRI site. The cloned gene was further characterized by colony PCR, restriction enzyme digestion and sequence analysis. The recombinant plasmid was transferred into GS115 strain of P. pastoris cells by electroporation. The His⁺ Pichia transformants were analyzed for the presence of the insert in the yeast genome by PCR. PCR positive clones were grown and expression was induced with 0.5% methanol. The expressed gene products were then characterized by SDS-PAGE and Western blot analysis. This is the first report on the production of FMDV non structural proteins in yeast. The expressed protein will be of diagnostic importance.

Keywords: 3AB protein, expression, FMDV, Pichia pastoris
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Introduction

Foot and mouth disease virus (FMDV) causes a devastating disease in cattle, pigs and sheep. The disease can spread rapidly among susceptible populations and has a great impact on the economy of the affected countries. Where the disease is sporadic, control of the disease is by slaughtering the affected and in-contact animals, whereas in enzootic areas, the control is by regular vaccination and restricted animal movement. Animals recovered from the FMD can become persistent carriers of the virus, in which case the subjects carry the virus for several years. The carrier animals harboring the sub clinical infection may be a source for new outbreaks. It is possible that these carrier animals intermittently shed variants of the virus, which differ antigenically from the original strain. Vaccination, thus, may be less effective in these animals due to the variant viruses. Detection of animals exposed to the disease in the livestock population is very essential for effective FMD control programmes. In fact, detection of replicating FMDV in the vaccinated animals not manifesting clinical signs is as relevant as the diagnosis of the acute infection itself, since it acts as an indicator for vaccine performance.

In endemic countries, like India, where regular vaccination is carried out in the susceptible population, majority of the susceptible animals are sero-positive for structural proteins and it is not possible to determine whether the animal is exposed to the disease using whole virus as antigen. Since non-structural proteins (nsps) are not present in vaccine preparations, detection of antibodies to these proteins is of diagnostic value whether the animal/herd is exposed to the disease or not. Therefore, the present requirement is to develop a serological test capable of differentiating vaccinated from the FMDV infected animals. Such a test would not only be useful to detect viral persistence but also for serological surveys to be carried out for FMD eradication. The test so developed should be highly specific and sensitive, simple, innocuous and inexpensive.

Nsps 2C, 3AB, 3ABC expressed in different expression systems have been used in the ELISA and other serological tests to detect the antibodies in infected animals. In endemic countries, like India, where regular vaccination is carried out in the susceptible population, majority of the susceptible animals are sero-positive for structural proteins and it is not possible to determine whether the animal is exposed to the disease using whole virus as antigen. Since non-structural proteins (nsps) are not present in vaccine preparations, detection of antibodies to these proteins is of diagnostic value whether the animal/herd is exposed to the disease or not. Therefore, the present requirement is to develop a serological test capable of differentiating vaccinated from the FMDV infected animals. Such a test would not only be useful to detect viral persistence but also for serological surveys to be carried out for FMD eradication. The test so developed should be highly specific and sensitive, simple, innocuous and inexpensive.

Nsps 2C, 3AB, 3ABC expressed in different expression systems have been used in the ELISA and other serological tests to detect the antibodies in infected animals. The recent reports have suggested that antibodies to 2C/3ABC/3AB could be the best serological indicator of infection with FMDV. However, these nsps produced in Escherichia coli showed poor reactivity with sera of infected animals probably due to poor solubility of the protein expressed in bacteria. Though the proteins produced in insect cells are soluble, maintenance of the insect
cells is laborious and costly. Yeast expression system, on the other hand, has several advantages over bacterial and insect cell systems, i.e. good over expression level, secretion of protein into the medium, ease of maintenance of the cells and scaling up production\textsuperscript{1}. The present study reports the expression of 3AB in the yeast, \textit{Pichia pastoris}.

Materials and Methods

Virus

FMDV serotype A (Ind 17/77) vaccine strain was grown in BHK-21 cl 13 monolayers. The seed virus was obtained from the FMD vaccine production laboratory of the Institute.

Sera Samples and Conjugate

Rabbit hyper immune serum against recombinant 3AB of FMDV expressed in \textit{E. coli} and rabbit anti-bovine IgG-HRPO conjugate, available in the laboratory, were used. Goat anti-rabbit IgG-HRPO conjugate was procured from Bangalore Genei Bangalore, India.

Host Strain and Plasmid Vector

\textit{P. pastoris} host strain, used in the study, was the histidine requiring auxotroph GS115 (\textit{His}4) pPIC-9K, whereas yeast transfer vector contained AOX1 promoter and transcription termination sequences along with MCS for insertion of the foreign genes of interest and wild type copy of the histidinol dehydrogenase (\textit{His}4) gene for selection of \textit{P. pastoris} transformants. Both GS115 (\textit{His}4) host strain as well as plasmid pPIC-9K were procured from Invitrogen, USA.

Amplification and Cloning of 3AB

Total RNA was extracted from infected BHK-21 cell culture supernatant using Trizol reagent (Invitrogen, USA). The cDNA copy was synthesized using the purified RNA as a template and 3dt (5' dTTTTTTAAGAAAGGAAG-OH3') as a primer with M-MLV (Molony Murine Leukemia Virus) reverse transcriptase as per standard procedure. The 3AB sequence was amplified from the cDNA using 3Aln (5'd-GGT GAT TGA CCG GGT TGA G-OH 3') and VPgR (5'd- GAC TAT CGA ATT CTT AGC TTT CTC-OH 3') as upstream and downstream primers, respectively. All the primers were designed based on the published FMDV A12 sequence data\textsuperscript{7}. A 50 \textmu L reaction mix containing 1.5 mM MgCl\textsubscript{2}, 100 \textmu M each of dNTPs, 25 mM Tris-HCl, 50 mM KCl, 20 pmol of each primer and one unit of \textit{Taq} DNA polymerase was amplified in the DNA Thermal Cycler 9600 (Perkin-Elmer Cetus, USA) with initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min with a final extension at 72°C for 10 min.

The amplified PCR product corresponding to 3AB (672 bp) gene was purified using wizard PCR prep DNA purification system (Promega, USA), digested with EcoRI and the digested product (589 bp) was cloned into the pPIC-9K at EcoRI site. The recombinant colonies grown on kanamycin plates were initially screened by colony PCR, using vector specific 5'AOXI (5'dGACTGGTTCCATGACAAGC-OH3') and insert specific VPgR (3'end of the insert) primer to check orientation of the insert, followed by insert release from the recombinant plasmid DNA. The recombinant plasmid with correct orientation of the insert (pPICA\textsuperscript{22}-3AB) was used for further study.

Sequencing of 3AB Gene

The 3AB insert in plasmid (pPIC-9K) was sequenced with 3'AOXI (5'dGCAATGGCATT-CCTGA-OH3') and 5'AOXI primers using the ABI 377 automated DNA sequencer (ABI Inc., USA) to confirm the frame and specificity of the cloned fragment. The sequence reaction was carried out using 1 \mu g of plasmid DNA and 2 pmol of the primer. Sequence data obtained was analyzed with the help of Omiga 1.13 DNA analysis software. Both nucleotide and derived amino acid sequences were compared with A12 sequence.

Expression of Cloned 3AB in \textit{P. pastoris}

The pPICA\textsuperscript{22}-3AB plasmid was linearized with \textit{SalI} and transferred into GS115 strain of \textit{P. pastoris} by electroporation for 10 msec with field strength of 7500 V/cm using Gene Pulsar II (Bio-Rad, USA). Transformants harbouring the plasmid-borne \textit{His}4 marker were selected on minimal plates lacking histidine. Yeast chromosomal DNA was extracted from the transformants (Mut+) by spheroplasting with zymolyase, followed by phenol:chloroform extraction.

Presence of the insert in the yeast genome was confirmed by PCR amplification using 5' AOX1 and 3'AOX1, vector specific primers. Induction of the protein expression was carried out by standard procedures\textsuperscript{8}. Single colony of the \textit{His'Mut}+ positive clone and the vector transformant of \textit{Pichia} was inoculated in 25 mL buffered glycerol-complex
medium (BMGY), kept in two 250 mL flasks, and incubated at 30°C in a shaking incubator (250-300 rpm) to reach an A600 of 6. The cells were harvested and suspended in buffered methanol-complex medium (BMMY) to an A600 of 1.0 (about 100-200 mL medium) and incubated at 30°C, and methanol was added to a final concentration of 0.5% at every 24 h interval up to 96 h. After 96 h incubation, entire culture supernatant was harvested and the secreted proteins were precipitated with 50% ammonium sulfate, and dialyzed against phosphate buffered saline (PBS, pH 7.4). The protein content was estimated by Bradford method\(^{11}\) with bovine serum albumin (BSA) as the standard. Partially purified proteins, from the recombinant \textit{Pichia} clone along with control pPIC-9K were separated on 15% polyacrylamide gels under denaturing conditions (SDS-PAGE) as per the method of Laemmli\(^{12}\).

A similar duplicate gel was blotted onto a PVDF membrane for immuno detection. The recombinant protein was detected by treating with anti-3AB rabbit serum (at 1:800 dilution), followed by an anti-rabbit antibody HRPO conjugate (1:1000) and orthodianisidine dihydrochloride (ODD) as substrate\(^{11}\).

\section*{Results}

\subsection*{Cloning of 3AB in Yeast Transfer Vector}

The 3AB-coding sequence was amplified by RT-PCR using RNA extracted from the virus, which resulted in the amplification of specific PCR product of 670 bp (Fig. 1, lane 2). The amplified product was purified, digested with \textit{EcoRI} and ligated with \textit{EcoRI} digested pPIC-9K vector. Presence of the insert (containing 3B and truncated 3A) in the recombinant plasmids was confirmed by \textit{EcoRI} digestion. Release of 0.6 kb fragment confirmed the presence of the insert. Orientation of the insert in the plasmid was confirmed by PCR with 5′-AOX1 (vector specific) and VPgR (virus specific) primers. Amplification of 1 kb product (Fig. 2, lane 1) showed that the insert is in 5′-3′ orientation whereas amplification of 0.4 kb product indicated the 3′-5′ orientation (Fig. 2, lanes 2 & 3).

\subsection*{Sequencing of 3AB Clone}

Nucleotide and the amino acid sequence of cloned 3AB gene of serotype A (Indian vaccine strain) is shown in Fig. 3. The cloned 3AB gene is of 672 nucleotides and codes for 224 amino acids. After digestion with \textit{EcoRI}, the PCR fragment (589 bp) was cloned into pPIC-9K, a yeast transfer vector, and

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Agarose gel electrophoresis of amplified PCR products: lane 1, Negative control (without cDNA); lane 2, PCR product (3AB) amplified from cDNA; & lane M, Standard DNA molecular weight marker (100bp ladder, Invitrogen, USA).}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Agarose gel electrophoresis of PCR products amplified from recombinant plasmids with 3AB inserts: lane M, Standard DNA molecular weight marker (100bp ladder Invitrogen, USA); lane 1, Amplified PCR products from the plasmid with 3AB insert in correct orientation; lanes 2 & 3, Amplified PCR products from the plasmids with 3AB insert in reverse orientation; & lane 4, pPIC-9K vector control.}
\end{figure}
frame and orientation of the insert was confirmed by sequencing. The insert was in frame with vector coded ATG codon. The sequence of the cloned insert and the deduced amino acid sequence translated in +3 frame as shown in Fig. 3. To examine the relation with reported sequence of the serotype A (A12 strain), nucleotide and the amino acid sequences of both the strains were aligned (Fig. 4), which indicated a homology of 90% in the nucleotide sequence and 96% in amino acid sequence, confirming that the sequence cloned was of 3AB gene. Variation in the amino acid sequence was more pronounced in 3A compared to the 3B protein.

Transformation of pPICA_{22}-3AB into *Pichia pastoris*

In order to express the 3AB gene, recombinant plasmid (pPICA_{22}-3AB) was linearized with *SalI* and was transferred into GS115 strain of *Pichia* by electroporation with linearized pPIC-9K vector as control. The recombinant *Pichia* clones were screened for confirming the presence of 3AB insert in the yeast genome by PCR using vector specific primers. Histidine positive clones with at least one integrated copy of the expression cassette can be easily distinguished from un-transformant colonies by comparing the size of PCR products amplified in PCR colony using the 5’AOX1 and 3’AOX1 primers. The
chromosomal DNAs from the individual clones were prepared and subjected to PCR. Amplification of specific 1.1 kbp product (corresponding to 612 bp insert and rest is from the vector) could be seen in the gel (Fig. 5, lanes 1 & 2). Of the five colonies screened by PCR for integration, two were found to carry the vector with 3AB insert, which were used for protein expression.

Expression of 3AB in *Pichia pastoris*

The PCR positive *Pichia* clones were grown separately and the expression was induced with 0.5% methanol. Since the inserted gene is present downstream to the secretary signal sequences, the expressed gene product was expected to be secreted out in the medium. Proteins expressed by recombinant clones carrying integrated DNA with and without insert in the culture supernatants were analyzed by SDS-PAGE, followed by Western blot analysis. A protein band of 26 kDa was seen in the gel (Fig. 6, lane 3) corresponding to 3AB protein. Protein bands separated in the gel were transferred on to PVDF membrane and detected with antiserum specific to 3AB (Fig. 7). An intensive color reaction was observed with the protein band size corresponding to 26 kDa from recombinant clone (Fig. 7, lane 1) which confirms the expression of 3AB protein by *P. pastoris*.

**Discussion**

In India, diagnosis based on the detection of antibodies to viral nsps is important to assess the prevalence of endemic disease. The antibodies against
virus capsid show the sero-conversion in the vaccinated animals, while the presence of antibodies against nsps shows the disease prevalence situation either due to previous out breaks or presence of the residual live virus in case of vaccinated animals. Diagnosis of infection at sub-clinical level is very much relevant both in the control and eradication of FMD in endemic areas and as a supportive measure to the ‘stamping out’ policy in FMD-free areas. The poly protein 3AB and 3ABC have been proposed as the most antigenic of the FMDV nsps and it has been argued that antibody specific for these proteins could be the most useful marker of viral replication. Hence, presence of antibodies against these nsps certainly indicates viral multiplication in the animal. Recently, the yeast has emerged as a powerful heterologous expression system for the production of high levels of functionally active recombinant proteins. To expedite such approaches that circumvent several problems encountered in expressing the 3AB, we expressed 3AB protein in yeast P. pastoris system. The cloning of the 3AB sequence downstream to the highly inducible AOX1 promoter and signal peptide permitted high-level expression and secretion of the recombinant protein. The translated product is in the form of a fusion protein, which gets secreted out after cleavage of the signal sequences. Using SalI linearized pPICA22-3AB plasmid in Pichia, homologous recombination between the transforming DNA and region of homology within the yeast genome is generally expected to occur within the His4 locus, which leads to the generation of Mut+ recombinant of GS115 cells. Electroporation method of transformation in Pichia yielded $2 \times 10^3$ His+ transformants/µg of DNA, which is less than reported in Saccharomyces $(10^3-10^4/µg$ of DNA).
The quantity of protein expressed by the recombinant yeast clone was 200 mg/L culture supernatant, which is in the range of 6.3 mg to 12 g/L culture depending on the nature of the protein as reported by various groups. The calculated size of the 3AB comes to 23 kDa, which is in agreement with the protein size observed in SDS-PAGE. This indicates that the product is from the cloned gene.

In conclusion, the results show the expression of 3AB gene of FMDV serotype A vaccine strain in *P. pastoris*, which will be useful for development of diagnostic test to differentiate the vaccinated from the infected animals.

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References