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Cloning of fusion (F) protein gene of peste des petits ruminants virus (PPRV) in secretory *Pichia pastoris* vector

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ABSTRACT

With advent and development of DNA recombinant technology and advantages of *p. pastoris* expression system, fusion (F) protein of PPRV expression, because of effective immunodominant role could be an appropriate candidate for production of recombinant vaccine against PPR disease. In this study, F gene of PPRV Nigeria 75/1 strain (1637 bp) was amplified using RT-PCR and purified. It was then cloned into pPICZαA a secretory expression vector of *P. pastoris* for first time. The insertion was proved by both production of a 218 bp segment in Nested PCR and isolation of gene from construct by restriction enzyme *(XbaI)*. Finally, It was sequenced. In conclusion, after the expression of fusion (F) gene in *p. pastoris* expression system, it can be used in production of recombinant vaccine against PPR disease.

Keywords: Peste des petits ruminants virus (PPRV), Fusion protein, Cloning, *P. pastoris*, Yeast expression vector

INTRODUCTION

Peste des petits ruminants virus (PPRV) causes high mortality in sheep and goats, leading to serious socio-economic problems (Perry *et al* 2001), The PPRV infections are widespread in Sub-Saharan Africa, Middle-East, Southern Asia, Iran and the Indian sub-continent (Diallo *et al* 2007). The disease is characterized by fever, oculonasal discharges, necrotizing and erosive stomatitis, enteritis and pneumonia (Balamurugan *et al* 2006).

The PPRV is classified in the Morbillivirus genus of the Paramyxoviridae family (Barrett, 2001) and is antigenically closely related to rinderpest virus (RPV) (Gibbs *et al* 1979). The genome of PPRV consists of a single-stranded, negative-sense RNA of 15.948 kb, encoding eight proteins in the order of 3′ N-P/C/V-M-F-H-L 5′ (Bailey *et al* 2005). For the control of PPR, vaccines have been developed or are on the development, following the same strategy as for the other Morbilliviruses. The first vaccine was an attenuated cell culture rinderpest vaccine as a heterologous vaccine (Taylor *et al* 1979). Vaccinated animals despite of resistance to PPRV

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challenge had not produced neutralizing antibodies against PPRV. This indicates that probably some replication of PPR challenge virus in the vaccinated animals and its transmission to in-contact animals occur (Taylor *et al* 1990). In 1989, a homologous vaccine was prepared by attenuation of PPRV strain Nigeria 75/1, through serial passages on Vero cells (Diallo *et al* 1989). This vaccine could prevent the transmission of challenge virus to in-contact animals and is presently permitted for immunization of sheep and goats against PPRV infections (Diallo, *et al* 2007). The thermo sensibility of PPRV vaccine, some reports of transient immuno - suppression induced by the virus (Rajak *et al* 2005), tendency for production of multivalent vaccines (Hosamani *et al* 2006) and a need for implementation of combined programs of vaccination and serosurveillance in endemic areas, have encouraged continuing research on the development of sub-unit vaccines. As the candidates for subunit vaccine preparation, paramyxoviruses contain two immunodominant integral membrane glycoproteins, namely, hemagglutinin (H) or hemagglutinin-neuraminidase (HN) and a fusion protein (F).The H/HN glycoprotein is the virus attachment protein, while the F protein is believed to disrupt the target cell membrane leading to virus-cell and cell-cell fusions. These surface glycoproteins, especially fusion proteins of morbilliviruses are highly immunogenic and confer protective immunity. To contribute in developing a suitable subunit vaccine against PPRV infection, the present work was undertaken for the first time to clone the full length F gene coding sequence of PPRV in pPICZαA, a secretory expression vector of yeast *P. pastoris* (Invitrogen).

MATERIALS AND METHODS

Virus Strain. Vero cell line at passage level of 21 was used for propagation of the vaccinal strain of PPRV (Nigeria 75/1) as described previously (Sarkar *et al* 2003). Viral aliquots of 1 ml were prepared and stored at -70 ˚C until use.

RT-PCR amplification of PPRV F gene. Viral RNA was extracted by High Pure Viral Nucleic Acid Kit (Roche), as per the manufacturer instruction and used as a template for F gene amplification. The oligonucleotides primers used in RT-PCR, were designed according to the PPRV Nigeria 75/1 sequence, available in the Genbank database (Accession Number: Z37017), and multiple cloning site of pPICZαA secretory plasmid of *P. pastoris* expression system (Invitrogen). The sequence of RT-PCR primers and a primer pair used in a confirmatory nested PCR are shown in table1. RT-PCR was performed by a one step RT-PCR kit (One Step-Titan kit, Roche) with a thermal cycling program of 45 ˚C for 45 min to prepare the cDNA, followed by 94 ˚C for 2 min and 35 amplification cycles of 94 ˚C for 30 sec, 55 ˚C for 1 min and 68 ˚C for 2 min. The final post polymerization step was 10 min at 72 ˚C.

Nested PCR. The specificity of the DNA fragment amplified in RT-PCR was confirmed by a nested PCR. The reaction was performed in a volume of 50 µl consisting of 2 mM Mgcl2, 10 mM dNTPs, 10 pmol of each primer (table 1) , 2 µl of purified RT-PCR product، 5µl of 10X PCR buffer and 1 unit of Taq DNA polymerase. Thermal program of nested PCR was as following: 3 min at 94 ˚C، 30 cycles of 94 ˚C for 30 Sec, 55 ˚C for 60 Sec and 72 ˚C for 2 min. The final post polymerization step was 10 min at 72 ˚C.

Cloning of F gene into pPICZαA vector. The RT-PCR Product of F gene was firstly cloned in pTZ57R/T vector of a T/A cloning system (InsT/AClone^{TM} PCR Product Cloning Kit, Fermentas) based on the manufacturers Protocol. After transforming competent *E. coli* TOP10F′ bacterial cells by the construct pTZ57R/T F, representative clones were tested for the correct size of the insert (F gene), then was sent to (MWG,

Germany) DNA sequencing by universal M13 forward and reverse primers (table 1). The construct pTZ57R/T F was then digested by *XbaI* restriction enzyme which recognition site has been added to the 5' ends of primers used in RT-PCR. After purification from agerose gel, F gene, digested with XbaI, was ligated to pPICZαA vector, previously digested with *XbaI*. *E.coli* TOP10F′ cells were transformed with the resulting legation product and plated on low salt LB/Zeocin medium containing 1%Trypton, 0.5% Yeast Extract, 0.5% Nacl, pH 7.5 and 25µg/ml Zeocin (Leber *et al* 1999). The resulting transformants were tested by restriction analysis and nested PCR. In order to confirm the integrity of F gene insert and that no errors were introduced at the legation sites, the construct pPICZαAF was purified from the bacterial colonies and complete sequence of F gene, inserted in pPICZαA was also determined by sequencing from two directions by vector specific primers (AOX1 forward and reverse primers, Table 1).

Table 1. List of PCR primers.

No	Primer	Sequence (5´to 3´)	Expected Size of the product (bp)
1	PPRF-for	GTA TCC TCT AGA ATG ACA CGG GTC GCA A	1673
2	PPRF-rev	GTA TCC TCT AGA CTA CAG TGA TCT CAC GTA	
3	NestPPRF-for	AGG CAC CAA TTT AGG CAA TG	218
4	NestPPRF-rev	TGA TTT TGG AGG CAG GAA TC	
5	AOX1-for	GAC TGG TTC CAA TTG ACA AGC	
6	AOX1-rev	GCA AAT GGC ATT CTG ACA TCC	
7	M13-uni for	TGT AAA ACG ACG GCC AGT	
8	M13-uni rev	CAG GAA ACA GCT ATG ACC	

RESULTS

The F gene coding region (5527–7164 position) of PPRV, strain Nigeria 75/1, was successfully amplified in RT-PCR with designed primers (Figure1). Specificity of the PCR product was shown by a nested PCR resulting in the amplification of a DNA fragment of 218bp (Figure2).

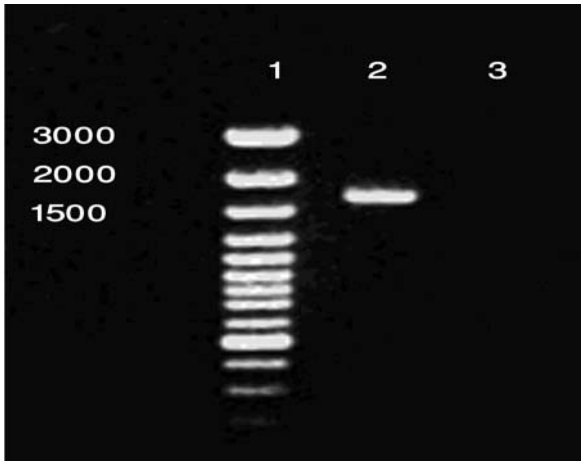

Figure1. PCR product of amplified F gene. *Lane 1:* Ladder (100 bp); *Lane 2:* Amplified F gene (1637 bp); *Lane 3*: Negative control.

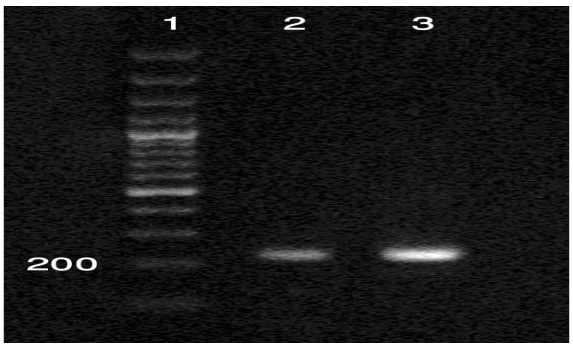

Figure2. Nested PCR of F gene. *Lane 1:* Ladder (100 bp); *Lane 2:* Amplified F gene (218); *Lane 3:* Construct (F + pPICZαA).

Purified PCR product (1637 bp) was then legated into pTZ57R/T and used for transformation of competent *E. coli*, strain TOP10F′. Digestion of purified plasmids from the bacterial colonies, with *XbaI*, confirmed the correct size of the insert (Figure3). As demonstrated in figure 4, digestion produced two DNA fragments of about 3000 and 1665 bp, corresponding to linearized vector and insert, respectively. To further confirm the identity of insert, complete sequence of F gene, inserted in pTZ57R/T was determined by sequencing from two directions by the vector specific primers. Alignment of the insert sequence with the original sequence of F gene of PPRV strain Nigeria 75/1 did not show

significant differences between the two sequences (Figure 5).

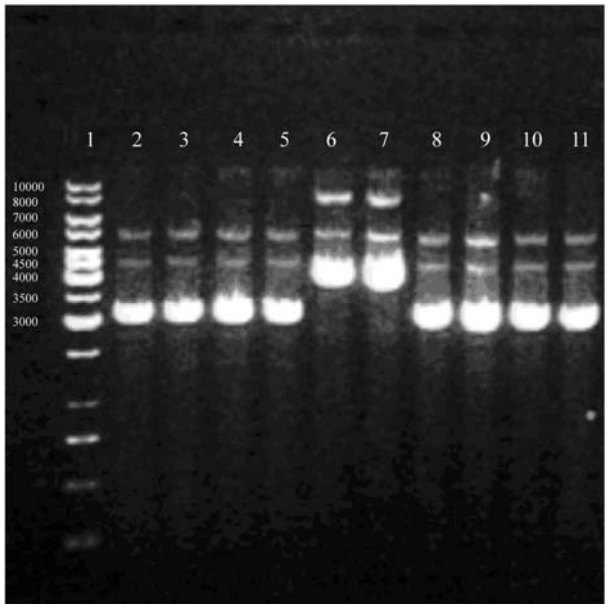

Figure3. Construct (F + pPICZαA) agarose gel electrophoresis. Lane1: ladder (1Kb); Lane 2-5 and 8-11: vectors free from insert; Lane 6 & 7: Constructs (F + pPICZαA).

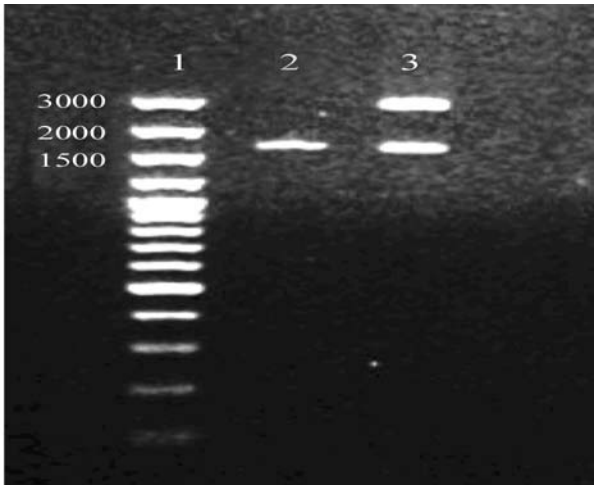

Figure4. Agarose gel electrophoresis of construct (F + pPICZαA) digested by *XbaI* . Lane1: ladder (100bp); Lane2: Positive control (F gene); Lane 3: digested construct with *XbaI*..

DISCUSSION

PPR is an economically significant disease of small ruminants (Dhar *et al* 2002) with an increasing global incidence (Nanda *et al* 1996, Ozkul *et al* 2002 and Shaila *et al* 1996). Despite the application of a live attenuated vaccine against the disease, researches have been continued to develop new generation of vaccines, especially due to the thermo sensibility of the live vaccine and for differentiation between infected and vaccinated animals (Diallo *et al* 2007). So far, the surface H/HN and F glycoproteins of several morbilliviruses have been expressed in various vector systems. Fusion Proteins of morbilliviruses are especially highly immunogenic and confer protective immunity (Diallo *et al* 2007). With regard to PPRV, attempts have been made to prepare subunit vaccines, with focusing on F and H/HN proteins of PPRV or RPV (Jonse *et al* 1993, Romero *et al 1995*, Sinnathamby *et al* 2001). PPRV is antigenically closely related to rinderpest virus (RPV) (Taylor, 1979a) and a Vaccinia virus, expressing H and F glycoproteins of RPV has been shown to protect goats against PPR disease (Jones *et a*l 1933). Capripox virus recombinants expressing the proteins H or F of RPV or the F protein of PPRV have also conferred protection against PPR disease in goat (Romero *et al* 1995). In this study, we amplified the F protein gene of PPRV strain Nigeria 75/1 by RT-PCR and cloned, in order to express the protein in *P. pastoris* expression system (invitrogen). Like in other morbilliviruses (Bailey *et al* 2005, Dhar *et al* 2006), F protein gene is one of the most conserved genes of PPRV. This has been shown by alignment of full length genome sequences of vaccinal and wild-type strains of PPRV (Bailey *et al* 2005). F protein is composed of a bout 546 amino acids with a molecular weight of about 59.310 KDa. The protein is synthesized as an inactive precursor (F0) which is subsequently cleaved by host cell enzymes to yield an active disulphide linked protein (F_1-F_2) on the surface of the cells (Bailey *et al* 2005). F_2 portion of the protein is glycosylated in 3 regions (lamb & parks 2007). We amplified F gene coding region (5527 –

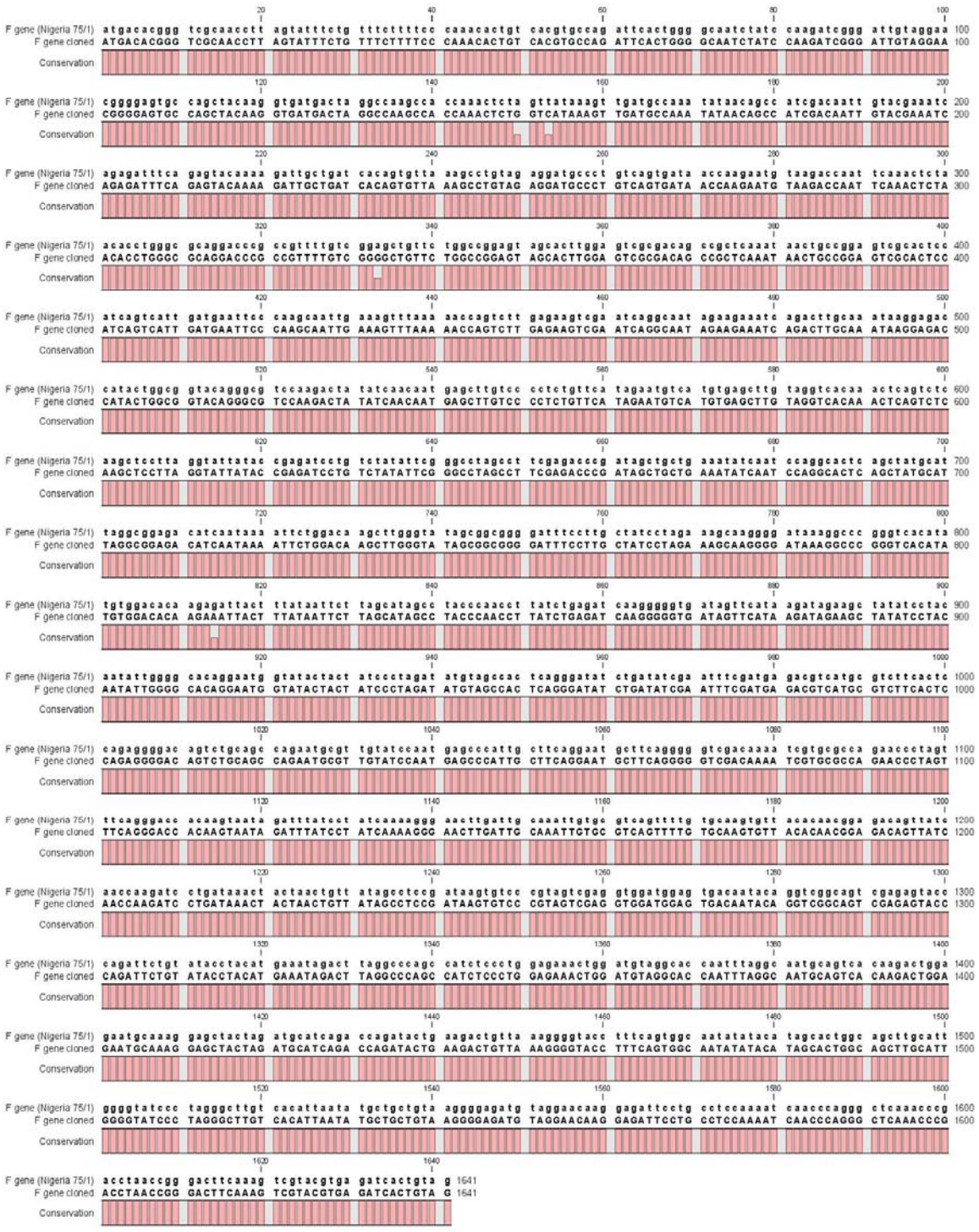

Figure5. Alignment of the insert sequence with the original sequence of F gene of PPRV strain Nigeria 75/1.

7164 position) of PPRV, strain Nigeria 75/1. Three silence mutations were noticed at positions 150, 153 and 333. Only one mutation, leading to an amino acid change (aspartic acid to asparagine), was found at position 814. Sequence analysis of the F gene cloned in pTZ57R/T and the F gene of PPRV

strain Nigeria 75/1, indicated that the mutation has occurred in a context of a random coil structure which was not abolished due to this mutation. Therefore, it's expected that the mutation would not affect the antigenic nature of the F protein. The F gene sequence excised from the construct pTZ57R/TF was subsequently legated into PPICZαA vector and sequenced after cloning in TOP10F′ strain of *E. coli*. The results indicated the accuracy of joining sites. *P. pastoris* is a methylotrophic yeast, exploited for high level expression and functionally active, heterologous proteins (Sreekrishna *et al* 1988, Laufer *et al* 2002, Sun 1997, Treerattrakool *et al* 2002). Therefore, we expect the construct PPICZαAF will be useful for production of F protein of PPRV in the yeast system.

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