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Research Article

Cloning and Expression of Highly Pathogenic Avian Influenza Virus Full-Length Nonstructural Gene in *Pichia pastoris*

M. B. Abubakar,[1] I. Aini,[2,3] A. R. Omar,[1,3] and M. Hair-Bejo[1]

[1] *Virology Laboratory, Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, Selangor 43400, Malaysia*
[2] *Department of Veterinary Clinical Studies, Faculty of Veterinary Medicine, Universiti Putra Malaysia, Selangor 43400, Malaysia*
[3] *Institute of Bioscience, Faculty of Veterinary Medicine, Universiti Putra Malaysia, Selangor 43400, Malaysia*

Correspondence should be addressed to M. B. Abubakar, mustydvm@gmail.com

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Avian influenza (AI) is a highly contagious and rapidly evolving pathogen of major concern to the poultry industry and human health. Rapid and accurate detection of avian influenza virus is a necessary tool for control of outbreaks and surveillance. The AI virus A/Chicken/Malaysia/5858/2004 (H5N1) was used as a template to produce DNA clones of the full-length NS1 genes via reverse transcriptase synthesis of cDNA by PCR amplification of the NS1 region. Products were cloned into pCR2.0 TOPO TA plasmid and subsequently subcloned into pPICZαA vector to construct a recombinant plasmid. Recombinant plasmid designated as pPICZαA-NS1 gene was confirmed by PCR colony screening, restriction enzyme digestion, and nucleotide sequence analysis. The recombinant plasmid was transformed into *Pichia pastoris GS115* strain by electroporation, and expressed protein was identified by SDS-PAGE and western blotting. A recombinant protein of approximately ~28 kDa was produced. The expressed protein was able to bind a rabbit polyclonal antibody of nonstructural protein (NS1) avian influenza virus H5N1. The result of the western blotting and solid-phase ELISA assay using H5N1 antibody indicated that the recombinant protein produced retained its antigenicity. This further indicates that *Pichia pastoris* could be an efficient expression system for a avian influenza virus nonstructural (NS1).

1. Introduction

Avian influenza is a highly contagious viral disease of major concern to both the poultry industry and human health; the currently ongoing outbreaks of H5N1 since 1997 have been unprecedented with over 500 million birds destroyed and over 303 humans dead. This development has ignited a global fear of imminent Influenza pandemic. Even though, adoption of vaccination policy as a targeted control or prevention measures has been generally discouraged by the scientific community. However, the need to boost eradication efforts in order to limit the spread of infections and ensure safety against international trade embargoes and avoid economic losses couple with the recent advances in DNA technology has prompted a re-evaluation of both vaccine strategies and restrategising of diagnostic tool as an additional effort in the battling against highly pathogenic avian influenza control programme [1–3].

Recent advances in molecular DNA biotechnology of protein expression and production of recombinant viral protein using various expression systems are becoming popular, more acceptable, and efficient. The ease with which genes are manipulated to yield recombinant protein both for vaccines and diagnostics is fast revolutionalizing the industry.

Yeast has been endowed with the capacity for easy posttranslational modification of expressed polypeptides with ease. Their intracellular environment is more suitable for correct folding of eukaryotic virus protein [4]. The use of methylotrophic yeast system for foreign protein expression is increasing dramatically, since the advent of the first world licensed genetically engineered vaccine antigen for hepatitis

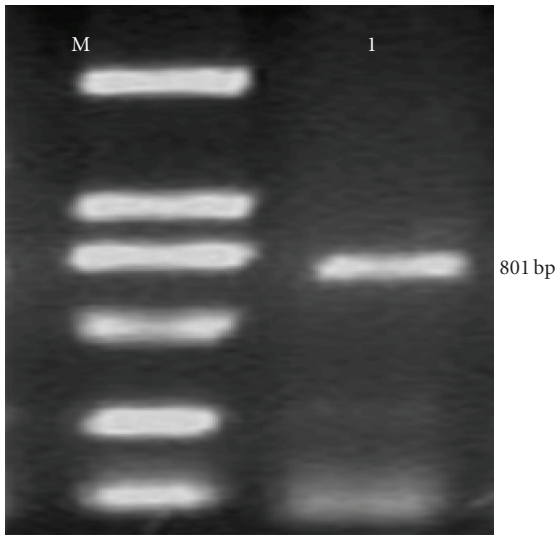


Figure 1: Agarose gel electrophoresis analysis of two-step RT-PCR amplified NS1 gene of AIV using NS1F and NS1R primers.

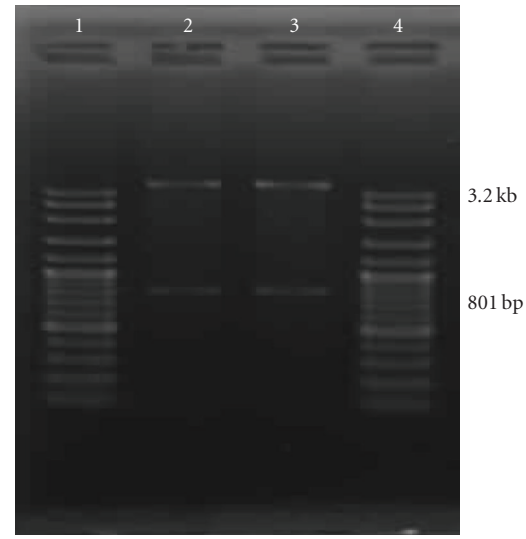


Figure 2: Restriction enzyme analysis of pPICZαA NS1 recombinant plasmid with XhoI and NotI in the Forward and backward primers, respectively; lanes 2 and 3: vector-3.2 kb and insert gene 801 bp, lane1: 1 kb DNA ladder marker (Fermentas, USA).

B for human in 1986 was prepared using yeast recombinant [5]. In this study, we described the expression of AIV NS1 in *Pichia pastoris* as an easy tool for generating efficient expression system that could have potential for serological diagnostics of AIV without compromising surveillance.

2. Materials and Methods

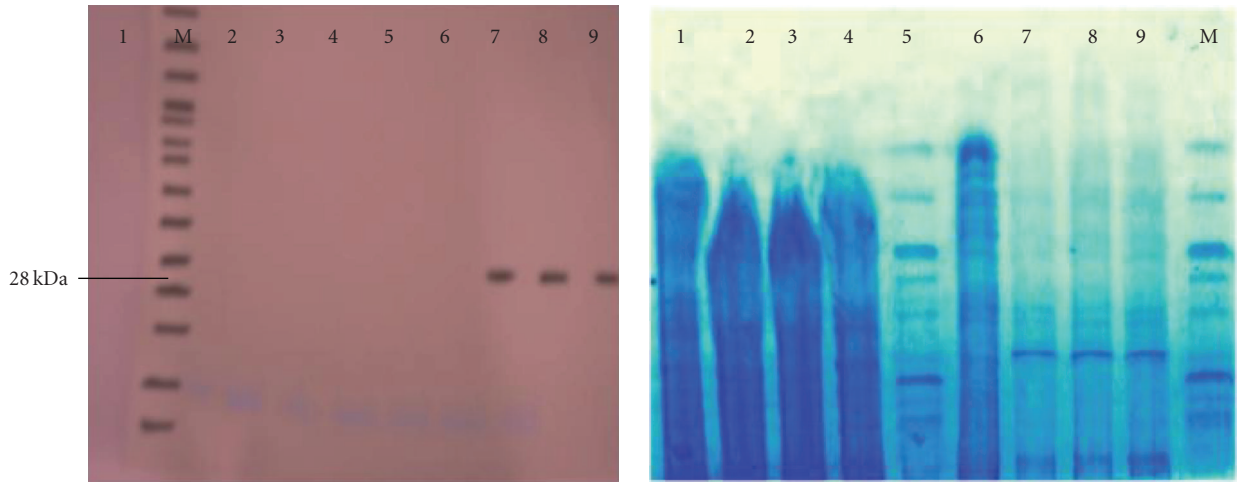
Inactivated influenza A/chicken/5858/Malaysian 2004 (H5N1) was kindly provided by Veterinary Research Institute, Ipoh, Perak, Malaysia. Trizol RNA extraction method was employed in the extraction of the viral RNA, under BLS-3 condition. Plasmid DNA extraction and gel purification were employed in accordance with kits manufacturer's instructions. The following primers were employed for RT-PCR: the sense primer P 1 and antisense primer F 5′CTC GGC TCG AGT ATG GAT TCC AAC ACT GTG 3′ and F 5′GAC TGC GGC CGC GCT CTT ATC TCT TGC TCC 3′. RT-PCR was performed under the following conditions: the reaction mixture contained in a final concentration of 1x of reaction buffer, 5 mM of $MgCl_2$, 0.8 μM of each primer (NS1 F & NS1 R), 0.1 mM of dNTP, 2 U of AMV reverse transcriptase, and 5 U of recombinant RNasin ribonuclease inhibitor and 100 ng of extracted RNA template. The mixture was initially incubated as follows: briefly, at 45°C for 50 min, reverse transcriptase denature at 94°C for 5 min, chilled at 4°C, the full denaturation at 94°C for 2 min, at 94°C for 1 min, 1 mins at 53°C, 2 min at 68°C for 40 circles of amplification, and 10 min at 68°C for an additional extension. The PCR products of the full length of AIV A/Chicken/5858/Malaysian/H5N1 strain NS1 generated was purified using GENEALL GEL SV Kit (General Biosystem, Korea) and subsequently cloned into pCR 2.1 TOPO vector from TOPO TA Cloning Kit (Invitrogen, USA) according to manufacturer's instructions. The amplified products of the gene encoding NS1 and pPICZαA expression vector were digested and subcloned according to standard procedures [6].

The recombinant pPICZαA-NS1 gene was sequenced by Macrogen Inc. (Korea), and nucleotide sequences were analyzed using Bio Edit software program. The sequence used for comparison or analysis in this study was A/chicken/Malaysia/5858/2004 (H5N1) (DQ321197.1) obtained from the GenBank database. The comparison was done using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The nucleotides of the NS1 gene were compared with the published sequences from around the world. A 100% homology was obtained with A/chicken/Malaysia/5858/2004 (H5N1) on NCBI blast (as shown below).

3. Results and Discussion

The full length of Influenza A/Chicken/Malaysia/5858/2004/(H5N1) NS1 gene of 801 bp in size was amplified by RT-PCR, sequenced, and cloned into pCR2.0 TOPO TA cloning vector (Figure 1). Purified DNA clone of amplified full-length NS1 gene was inserted into the expression vector pPICZαA to generate a recombinant plasmid pPICZαA-NS1 (Figure 2). After comparing the nucleotide sequence of NS1 gene derived from the recombinant plasmid pPICZαA-NS1, its deduced amino acid sequence was found to be NS1 of H5N1 strain with 100% homology, and the nucleotide sequence was found to be similar to that influenza A/Chicken/Malaysia/5858/2004 (H5N1) strain with 100% homology, as shown in (Figure 4).

Immunoblotting results revealed the antigenic band of ~28 kDa NS1 of HPAI using polyclonal antibody against the 28 kDa NS1 protein. These findings strongly suggested that this protein might be useful as potential antigen for diagnostics against natural/experimental HPAI infections. This result



(a) (b)

Figure 3: (a) Western blot analysis showing the expressed NS1 recombinant protein of ~28 kDa in expressed GS115 *Pichia pastoris strain* using NS1 polyclonal antibody as a primary antibody; expression started at day 7 after methanol induction; (b) SDS-PAGE separating the proteins into different molecular weight prior to transfer to NC membrane and immunodetection.

Table 1: OD_{650} values of preliminary ELISA for detection of positive NS1 recombinant protein.

Dilution	10	20	40	80	160	320	640	1280	2560	5120	10240	Control
*Positive	0.924	0.945	0.865	0.729	0.597	0.489	0.325	0.245	0.127	0.096	0.078	0.054
**Negative	0.083	0.080	0.072	0.059	0.051	0.047	0.034	0.030	0.031	0.026	0.334	0.032

* Positive purified cell lysate. **Negative purified cell lysate.

also indicated successful expression of NS1 gene of avian influenza subtype H5N1 designated as GS115/pPICZA/NS1, an approximately ~28 kDa protein confirmed via western blot using NS1 polyclonal antibody (Figure 3) and indirect ELISA using purified recombinant NS1 protein as coating antigen (Table 1).

While avian Influenza (AI) continues to remind us of one of the most fearful diseases threatening the poultry industry and human population, it has an economic burden and international trade impacts with serious embargoes; it causes loss of production and productivity both on individual and the society [7, 8]. NS1 of the influenza virus is a highly conserved gene when compared with other encoded proteins, though it has poor antigenicity. Previous study showed that within the influenza virion there was no NS1 protein, but it could be highly expressed by cells infected with influenza virus [9]. Therefore, birds vaccinated with inactivated vaccines did not produce nonstructural protein-specific antibodies, so NS1 antibodies are only presented in naturally infected birds; therefore, this could provide a "window" opportunity for the differentiation between the vaccinated and the naturally infected birds, a major challenge to serological surveillance. In this study, we amplified and cloned the NS1 gene of an AI strain A/Chicken/Malaysia/5858/2004 (H5N1), into the pPICZαA eukaryotic expression system. The recombinant plasmid designated as GS115-pPICZαA-NS1 was analyzed by RE digestion, PCR colony screening, and sequencing analysis. Immunodetection using NS1 polyclonal in antibody in western blot (Figure 3) further confirmed the antigenicity and correct folding of the recombinant protein. Restriction enzyme digestion has been a method of choice when screening a lot of transformants. However, high zeocin concentration were used for screening of high copy no of positive Pichia transformants [6]. In conclusion, the NS1 gene of H5N1 subtype influenza A virus was successfully cloned and expressed via shake flask following methanol inductions. These results laid foundation as a preliminaries finding for developing an effective diagnostics marker for influenza A (H5N1) using recombinant DNA technology.

The inability to differentiate antibody due to either natural infection or vaccination has been a serious setback hindering the progress of surveillance programme by interfering with accuracy of diagnosis, and by extension this can affect the free market flow of poultry and poultry product at the international market, resulting in a trade embargoes enforcement. In the present study, it has been demonstrated that the ability to clone and express NS1 recombinant protein in a shake flask fermentation condition will offer an armful opportunity for the production of this NS1 recombinant protein that was shown to specifically bind NS1 polyclonal antibodies on Western blot; it has also shown a potentially promising result in an indirect ELISA (Table 1) using putative recombinant as coating antigen. It could differentiate between infected and vaccinated birds, in that NS1 antibodies could only be detected in natural/experimental infections. Based on the above results, it has

>gb|DQ321197.1| Influenza A virus (A/chicken/Malaysia/5858/2004 (H5N1)) nonstructural protein (NS) gene, complete cds Length = 801
Score = 1445 bits (1602), Expect = 0.0
Identities = 801/801 (100%), Gaps = 0/801 (0%)
Strand = Plus/Plus

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Query  1    ATGGATTCCAACACTGTGTCAAGCTTTCAGGTAGACTGCTTTCTTTGGCATGTCCGCAAA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    ATGGATTCCAACACTGTGTCAAGCTTTCAGGTAGACTGCTTTCTTTGGCATGTCCGCAAA  60

Query  61   CGATTTGCAGACCAAGAACTGGGTGATGCCCCATTCCTTGACCGGCTTCGCCGAGATCAG  120
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Sbjct  61   CGATTTGCAGACCAAGAACTGGGTGATGCCCCATTCCTTGACCGGCTTCGCCGAGATCAG  120

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Figure 4

been clearly demonstrated that the difference between the positive and negative purified cell lysate samples is more than threefold difference; therefore, it is envisage that this may have a good potential for diagnosis in the case of clinical or experimental infections or natural outbreaks, even though this might require further assay using field samples. Previous studies had shown that a difference equal to or greater than threefold is enough to demonstrate the establishment of an infection, while the indirect ELISA is ongoing trials and validation have been projected that this could be useful at quarantine stations, where only a single convalescent serum sample is obtained. This technology would offer a simple and less expensive diagnostic antigen with less hazardous contact with a whole viral particle commonly encountered in the conventional method.

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