FULL PAPER

Virology

System for the heterologous expression of NS1 protein of H9N2 avian influenza virus in the ciliate *Tetrahymena thermophila*

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ABSTRACT. *Tetrahymena* is commonly used as an alternative eukaryotic system for efficiently expressing heterologous genes. In this study, we inserted the non-structural (*NS*) 1 gene of avian influenza virus (AIV) into the shuttle vector pD5H8 and transformed conjugating *T. thermophila* with the recombinant plasmid pD5H8-NS1 by particle bombardment. Positive transformants were selected with paromomycin. We demonstrated that the NS1 protein could be expressed steadily following induction with cadmium in this *Tetrahymena* system. An enzyme-linked immunosorbent assay detection method was preliminary established using the expressed protein as coating antigens for serodiagnosis. This is the first study in which a *Tetrahymena* expression system was employed for the expression of the AIV NS1 protein, and it provides a good basis for the development of differential diagnostic kits and vaccines for the prevention and control of avian influenza.

KEY WORDS: avian influenza, heterologous expression, *NS1* gene, *Tetrahymena*, virus

In recent years, avian influenza caused by the H9N2 subtype AIV has been widely prevalent among domestic poultry and wildfowl [16]. The H9N2 subtype AIV was first isolated from turkeys in 1966, but did not attract the attention of researchers. Since then, the virus has been detected in various species, including aquatic wild birds, ducks, chickens, horses, and pigs. It was not until 1997, when bird flu broke out in Hong Kong and was transmitted to humans, resulting in the death of six people, that public health concerns were raised [10]. Low-virulence H9N2 subtype influenza strains can become virulent after reassortment [11, 16]. Experts predict that emergence of new pandemic subtypes and the selective variation of viral antigens may be related to host immune pressure and vaccine usage. Some researchers have claimed that poultry carrying the H9N2 subtype AIV may serve as incubators for avian influenza virus infection in humans, increasing the risk of future pandemics [11].

Avian influenza A virus is a member of the Orthomyxoviridae family that possesses eight separate gene segments, the shortest being the non-structural (*NS*) gene segment. During the process of virus replication, the *NS* gene is translated into a collinear mRNA encoding two functionally important proteins: the 26 kDa NS1 protein and the 14 kDa nuclear export protein. The multifunctional NS1 protein contains two typical domains, the N-terminal RNA-binding domain and the C-terminal effector domain that binds several host proteins [3]. The NS1 protein plays a critical role in modulating the destructive power of AIV towards infected hosts, and is closely associated with AIV replication, pathogenicity, virulence, and host range [4, 14]. NS1 is a conserved protein that is secreted from infected cells and can be found in the serum at detectable levels [17]. The clinical applications of NS1 protein include differential diagnoses of avian influenza virus-infected field sera samples. New outbreaks of influenza viruses are highly variable, increasing the likelihood that new antigenic variants will arise. Therefore, early diagnosis is urgently needed to control and prevent future avian influenza epidemics. For the clinical detection of AIV antibodies, the ELISA method mainly uses inactivated AIV as the coating antigen. However, this method might increase the risk of spreading live viruses. Therefore, utilizing recombinant NS1 proteins as coated subunit antigens might be safer than using inactivated antigens.

In contrast to other eukaryotic expression systems, such as mammalian-based systems, the *Tetrahymena* expression system offers numerous other advantages, besides the expression of biologically active and functional proteins. *Tetrahymena* grow

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Table 1. Sequence of primers used in this study

Primer	DNA sequence
FQNS1F-*Bam*HI	*GGATCC*ATGGACTCCAACACTGTGTCAAG
FQNS1R-*Xho*I	*CTCGAG*TCAGTGATGGTGATGGTGATGTTTATCGTCGTCGTCTTTATAATCCTTTGGAGAGAGTGGAGGTC
pTIEV4-1206F	AATCATGAGTTCACCATTTAAAC
pTIEV4-1496R	AGCAATAACACCTTGAAGCAAAG
pD5H8-12476F	AACATGGAACGGGTATT
pD5H8-13086R	CTAAGCTCATTCTGCGTAAA

rapidly (2–2.5 hr per generation) and can be maintained at a lower cost (chemically defined media, free of peptides and serum components can be used) [1]. Researchers have developed advanced molecular genetic tools and techniques, including genetic recombination and transfection, specifically for *Tetrahymena*. The *Tetrahymena* system is well suited for the study of gene function using DNA-mediated transformation technology to overexpress transgenes [12]. The circular shuttle vector pD5H8 is an rDNA-engineered transgene expression vector that contains the high-activity metallothionein promoter [15]. A previous study [9] demonstrated that the viral protein hemagglutinin can be successfully expressed in a ciliate host. Thus, it is feasible to construct a transgene expression system using *Tetrahymena* for the stable expression of the NS1 protein of AIV isolated from domestic ducks. In this study, we inserted the *NS1* gene into the shuttle vector pD5H8 and transformed the recombinant plasmid pD5H8-NS1 into conjugating *T. thermophila* by particle bombardment. Positive transformants were selected with paromomycin. We demonstrated that the NS1 protein could be stably expressed following induction with cadmium in this *Tetrahymena* system. Our findings can potentially be used for the development of differential diagnostic kits and vaccines for the control of avian influenza.

MATERIALS AND METHODS

Ethical statement

All animal experiments in this study were approved by the Research Ethics Committee of the Fujian Academy of Agricultural Science. The animal experimental procedures were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of China. We adhered to the ethical guidelines for the care and use of animals.

Strains, plasmids, and serum samples

The domestic duck-origin strain [A/Duck/Fujian/FQ2/2007 (H9N2), GenBank No.: JF916709] was isolated and stored in our laboratory. The proliferation of this strain has been described previously [20]. Rabbit antiserum [A/Duck/Fujian/FQ2/2007 (H9N2)] was prepared using the traditional immunization method. The purified virus was emulsified with an equal volume of complete Freund's adjuvant or incomplete Freund's adjuvant, and the rabbit was immunized with 1 mg antigen by subcutaneous injection each time. The antiserum was collected after the fourth immunization. Preimmunization rabbit serum was collected before immunization. Newcastle disease virus (NDV)-positive sera were purchased from Harbin Veterinary Research Institute (Harbin, China). Sera against infectious bursal disease virus (IBDV) and duck Tembusu virus (DTMUV) were prepared by the poultry disease laboratory [6]. *T. thermophila* strains CU427 and CU428, the pTIEV4 vector, and the shuttle vector pD5H8 were all kindly provided by Professor Theodore G. Clark from Cornell University.

NS1 gene codon optimization and amplification

The *NS1* gene-specific primers for A/Duck/Fujian/FQ2/2007 (H9N2) strain were designed according to optimized nucleotides (Table 1). The forward and reverse primers carried the *Bam*HI and *Xho*I restriction sites, respectively. PCR assay was performed according to a previously described protocol [20] and the PCR products were electrophoresed on a 1.2% agarose gel. The target bands were excised and extracted using a Gel Extraction kit (Omega Bio-Tek, Norcross, GA, U.S.A.). The codon usage preference of the *NS1* gene and Tetrahymena was analyzed by the codon usage analyzer, http://gcua.schoedl.de/, with the Kazusa codon usage database, http://www.kazusa.or.jp/codon/. To the optimized *NS1* gene, we added a signal peptide at the 5′ end and a hexa-histidine tag at the 3′ end for purification. The whole sequence was synthesized by TaKaRa Co. (Dalian, China). The NS1 amino acid sequences were compared between the representative strains: A/Chicken/Beijing/1/94 (H9N2), A/Chicken/Fujian/G9/2009 (H9N2), A/Chicken/Fujian/25/00 (H9N2), and A/Chicken/Shangdong/6/96 (H9N2), and other H9N2 subtype strains: A/Chicken/Fujian/25/00, A/Chicken/Fujian/9290/2005, and A/Chicken/Fujian/25/00 isolated in Fujian using the ESPript program (http://esprit.ibcp.fr/ESPript/cgi-bin/ESPript.cgi).

Construction of transformation plasmid pD5H8-NS1

The cassette structure in pTIEV4 vector is shown in Fig. 1. The PCR products and pTIEV4 vector were digested with *Bam*HI and *Xho*I (New England Biolabs, Ipswich, MA, U.S.A.) separately. Then, the inserts and vector were mixed at a molar ratio of 6:1 and ligated overnight at 16°C with T4 ligase (Thermo Fisher Scientific, Waltham, MA, U.S.A.). The ligation products were transformed into competent *E. coli* DH5α cells and grown in Luria Bertani agar medium containing ampicillin 100 *µ*g/m*l*. pTIEV4-
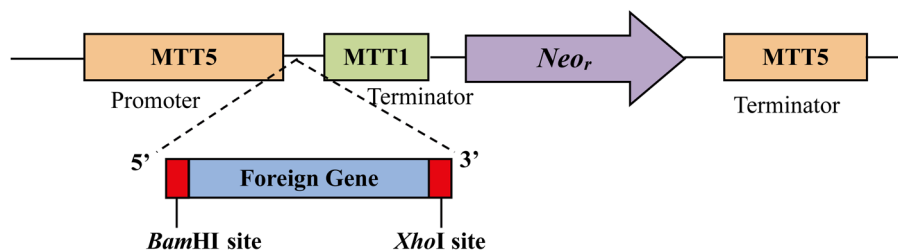
Fig. 1. Cassette structure in pTIEV4 vector.

NS1-positive colonies were identified by PCR using forward (pTIEV4-1206F) and reverse primers (pTIEV4-1496R) (Table 1). The pTIEV4-NS1-positive plasmid and the shuttle vector pD5H8 were digested with *Not*I. To avoid self-ligation of the vector, the pD5H8 shuttle vector was dephosphorylated with calf intestinal alkaline phosphatase (Thermo Fisher Scientific), according to the manufacturer's recommendations. After ligation at 16°C overnight, the ligation products were electroporated into *E. coli* HST08 electrocompetent cells (TaKaRa) using an electroporator (Bio-Rad, Hercules, CA, U.S.A.) at 300 V, 25 μF, and 50 Ω in 0.2 cm cuvettes. After electroporation, 1 ml of super optimal broth with catabolite repression (SOC, 2% tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5 mM KCl, 10 mM MgCl$_2$, 20 mM glucose) was added to the cuvette immediately to gently resuspend the cells. The cells were plated on Luria Bertani agar medium, containing 100 μg/ml of ampicillin. Single colonies were selected and identified by PCR using the primers pD5H8-12476F and pD5H8-13086R (see Table 1). *Not*I digestion was performed to identify the recombinants and sequencing was used to verify the direction of the inserted *NS1* gene. pD5H8-NS1-positive plasmid was extracted with an Endotoxin Plasmid kit (Omega Bio-Tek).

Construction and screening of recombinant T. thermophila

The *T. thermophila* strains CU427 and CU428 were cultured in Neff medium (0.25% proteose peptone, 0.25% yeast extract, 0.55% glucose, 33 μM FeCl$_3$, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B) with shaking at 80 rpm at 30°C to attain a logarithmic growth phase of 5×10^5 cells/ml. The growing cells were washed and suspended in 10 mM Tris-HCl (pH 7.4) in the original volume. After 24 hr of starvation, the cells were fixed with paraformaldehyde and then mixed together in equal quantities. Following a static culture for 10–11 hr, the mixtures were observed under a microscope for pair formation. When the mating rate was equal to 80%, biolistic bombardment transformation was carried out. The gold particles were coated immediately before use with recombinant plasmid (pD5H8-NS1) using a previously described method [1]. Meanwhile, cells were centrifuged at 800 g for 2 min, resuspended in 10 mM Tris-HCl (pH 7.4), and the whole cell sample was evenly spread on a sterile pre-wet filter paper and bombarded with the Biolistic PDS-1000/He particle delivery system (Bio-Rad) under stable vacuum (26 inches Hg). The transformed *T. thermophila* cells were then immediately transferred to Neff medium and cultured at 30°C without shaking. After 24 hr of culture, cells (100 μl cells/well) were plated on microtiter plates and 100 μg/ml paromomycin (final concentration) was added for selection. The concentration of paromomycin was gradually increased up to 200, 400, 600, 800, and 1,000 μg/ml every 48 hr. Viable monoclonal strains were cultured until a logarithmical growth phase of 5×10^5 cells/ml was reached, and then total genomic DNA was extracted for PCR amplification.

Expression and identification of NS1 protein

The recombinant *T. thermophila* strains were cultured at 5×10^5 cells/ml in Neff medium for 16 hr and treated with 15 μg/ml of cadmium chloride to induce transgene expression. *T. thermophila* cells were collected by centrifugation for 5 min at 1,000 g, and then resuspended in pre-chilled buffer A (40 mM Hepes, 1 mM CaCl$_2$, pH 7.4, and 20 μg/ml PMSF) and an equal volume of 600 mM NaCl. After centrifugation at 5,000 g for 3 min, the disrupted cells were separated into three layers, including the supernatant, proteinaceous gel, and precipitate. The proteinaceous gel and precipitate were washed in 10 volumes of buffer A and centrifuged at 5,000 g for 3 min. Finally, the samples were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting.

Protein samples were loaded onto a 12% SDS-PAGE mini-gel under reducing conditions. The separated proteins were transferred onto a nitrocellulose membrane for 2 hr at 45 mA using a semi-dry transfer system (Bio-Rad) and blocked with Tris buffered saline (TBS, 150 mM NaCl, 20 mM Tris-HCl, pH 8.0) containing 3% bovine serum albumin overnight at 4°C. The recombinant NS1 protein was detected in transformed *T. thermophila* using specific rabbit antiserum (1:100). After washing with TBST (TBS with 0.05% Tween-20), the membrane was incubated with secondary goat anti-rabbit IgG antibodies conjugated to alkaline phosphatase (1:20,000) (Sigma-Aldrich, St. Louis, MO, U.S.A.) for 1 hr at 37°C. The membrane was then rinsed extensively and developed using BCIP/NBT substrate solution, until the protein bands became visible. The target protein bands on the gel were excised, and enzymatically digested into peptides by trypsin for liquid chromatography tandem mass spectrometry (LC-MS/MS). The data were searched using the MASCOT program (http://www.matrixscience.com). The procedure for purification of recombinant protein has been described previously [9]. The protein was purified by metal affinity chromatography and then dialyzed overnight. Finally, the protein was concentrated to 6 mg/ml using a 10 kDa Amicon Ultra-15 centrifugal filter tube (Merck Millipore, Ireland). Protein purity was assayed by SDS-PAGE.

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protein       D   S   N   T   V   S   S   F   Q   V   D   C   F   L   W   H   V   R
Original 1    GACTCCAACACTGTGTCAAGCTTCCAGGTAGATTGTTTTCTTTGGCATGTCCGC
Optimized     GATAGTAATACTGTTTCTTCTTTTCAAGTTGATTGCTTTTTATGGCATGTTAGA

protein       K   R   F   A   D   Q   E   L   G   D   A   P   F   L   D   R   L   R
Original 55   AAACGATTTGCAGACCAAGAACTGGGTGATGCCCCATTTCTAGACCGGCTTCGC
Optimized     AAAAGATTTGCTGATCAAGAATTAGGTGATGCTCCTTTTCTTGATAGATTAAGA

protein       R   D   Q   K   S   L   R   G   R   G   S   T   L   G   L   D   I   R
Original 109  CGGGATCAGAAGTCCCTGAGAGGGAAGAGGCAGCACTCTTGGTCTAGACATCAGA
Optimized     AGAGATCAAAAATCTTTAAGAGGAAGAGGTTCTACTTTAGGATTAGATATTAGA

protein       T   A   T   R   E   G   K   H   I   V   E   R   I   L   E   G   E   S
Original 163  ACCGCAACTCGTGAAGGGAAGCATATAGTGGAACGAATTCTGGAGGGAGAATCA
Optimized     ACTGCTACTAGAGAAGGTAAACATATTGTTGAAAGAATATTAGAAGGTGAATCT

protein       D   E   A   L   K   M   T   I   A   S   V   P   S   P   R   Y   L   T
Original 217  GATGAAGCACTTAAAATGACTATTGCTTCAGTGCCATCTCCACGATACCTAACC
Optimized     GATGAAGCTTTAAAAATGACTATAGCTTCTGTTCCATCTCCTAGATATTTAACT

protein       D   M   T   L   E   E   M   S   R   D   W   L   M   L   I   P   K   Q
Original 271  GACATGACTCTTGAAGAAATGTCAAGAGATTGGTTAATGCTCATTCCCAAACAA
Optimized     GATATGACATTAGAAGAAATGTCAAGAGATTGGTTAATGTTAATTCCTAAACAA

protein       K   V   T   G   S   L   C   I   R   M   D   Q   A   T   M   D   K   T
Original 325  AAAGTGACAGGGTCCCTTTGCATTAGAATGGACCAAGCAACAATGGATAAAACC
Optimized     AAAGTTACAGGTTCTTTATGTATAAGAATGGATCAAGCTACAATGGATAAAACT

Protein       I   T   L   K   A   N   F   S   V   I   F   N   R   L   E   A   L   V
Original 379  ATCACATTAAAAGCGAACTTCAGTGTGATTTTCAATCGATTGGAAGCTCTAGTA
Optimized     ATAACATTAAAAGCTAATTTTAGTGTAATTTTTAATAGATTAGAAGCTTTAGTA

Protein       L   L   R   A   F   T   D   E   G   A   I   V   G   E   I   S   P   L
Original 433  CTACTTAGAGCTTTTACAGACGAAGGAGCAATAGTGGGTGAAATCTCACCATTA
Optimized     TTATTAAGAGCTTTTACTGATGAAGGAGCTATTGTTGGTGAAATTTCTCCTTTA

Protein       P   S   L   P   G   H   T   D   E   D   V   K   N   A   I   G   V   L
Original 487  CCTTCTCTTCCAGGACATACTGATGAGGATGTCAAAAATGCGATTGGGGTCCTC
Optimized     CCTTCTTTACCTGGTCATACTGATGAAGATGTTAAAAATGCTATTGGTGTTTTA

Protein       I   G   G   F   E   W   N   D   N   T   V   R   V   S   E   N   L   Q
Original 541  ATCGGAGGATTTGAATGGAATGATAACACAGTTCGAGTCTCTGAAAATCTACAG
Optimized     ATTGGTGGTTTTGAATGGAATGATAATACTGTTAGAGTTTCAGAAAATTTACAA

Protein       R   F   A   W   R   N   S   D   E   D   G   G   P   P   L   S   P   K
Original 595  AGATTCGCTTGGAGAAACAGCGATGAGGATGGGGGACCTCCACTCTCTCCAAAG
Optimized     AGATTTGCTTGGAGAAATTCAGATGAAGATGGTGGTCCTCCATTAAGTCCTAAA
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Fig. 2. The codon-optimized sequence of NS1 nucleic acid molecule. It is noteworthy that the gene sequence does not include the start codon ATG and the terminator codon TGA. Foreign genes with TGA would be correctly expressed. Differences in codon usage after optimization are indicated using red fonts.

Establishment of indirect ELISA detection

Microtiter plates (96-well) were pre-coated with 30 μg/ml of purified protein from a mucocyst gel layer in phosphate buffered saline (PBS) by incubating the plates with 50 μl of protein per well overnight at 4°C. The coated wells were blocked with PBS containing 2% bovine serum albumin (80 μl in each well) for 2 hr at 37°C. Rabbit antiserum or preimmunization rabbit serum (1:100; 50 μl per well) was added to each well for 1 hr at 37°C. The poultry sera against NDV, IBDV, and DTMUV were diluted 1:5 with PBST (PBS with 0.05% Tween-20) and 1% bovine serum albumin, and incubated under the same conditions. Each sample was measured three times and averaged. Horseradish peroxidase-conjugated goat anti-rabbit or goat anti-duck IgG secondary antibody (1:5,000; 50 μl per well; Sigma-Aldrich) was added to the plates and incubated for 1 hr at 37°C. The substrate *O*-phenylenediamine dihydrochloride was added after washing three times with PBST. The absorbance at 492 nm was measured using an MicroplateReader (Bio-Rad), and the sample was determined to be positive when P/N ≥2.1.

RESULTS

NS1 gene codon optimization and amplification

The total length of *NS1* gene [A/Duck/Fujian/FQ2/2007 (H9N2)] consists of 654 nucleotides, encoding 217 amino acids. We chose TGA to be the only terminator codon, since it has been reported that foreign (not ciliate) genes carrying TAA or TGA triplets as the terminator codon are not correctly expressed. A comparison between the viral *NS1* original sequence and codon-optimized *NS1* sequence for expression in *T. thermophila* is shown in Fig. 2. After optimization, the AT content changed from 55 to 70%
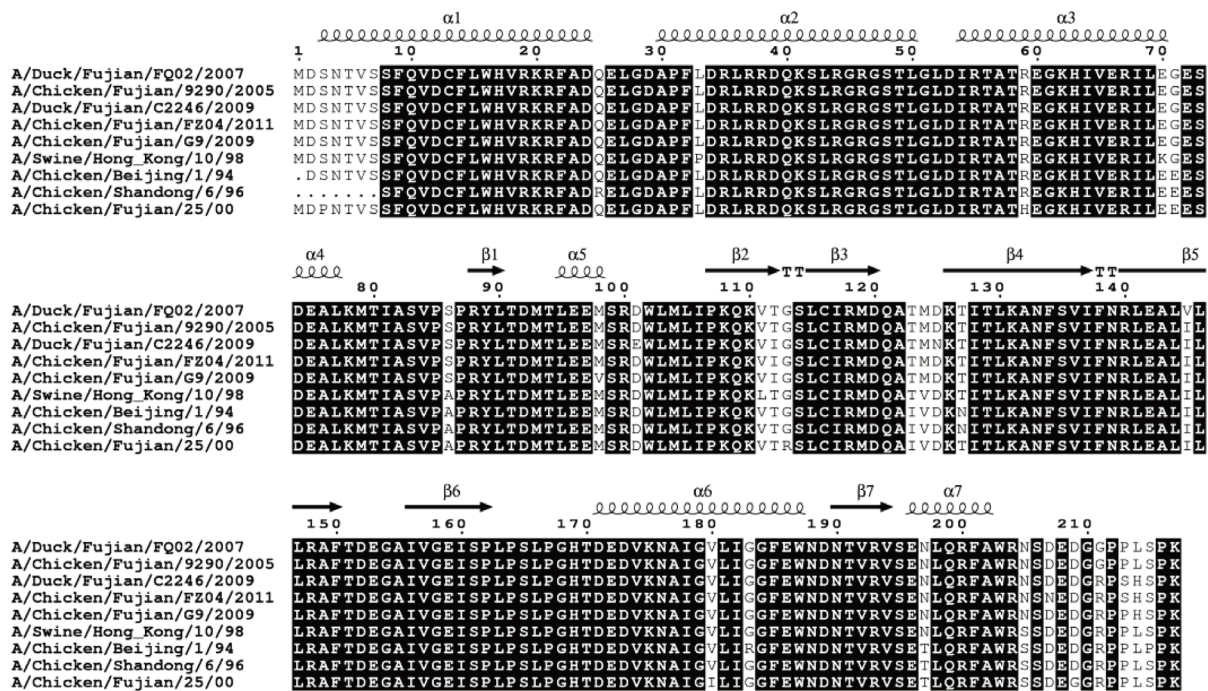
Fig. 3. Secondary structure and sequence alignment of NS1 proteins of different avian influenza viruses. Dots indicate deletion residues. Identical amino acids are shown as white letters in black boxes.
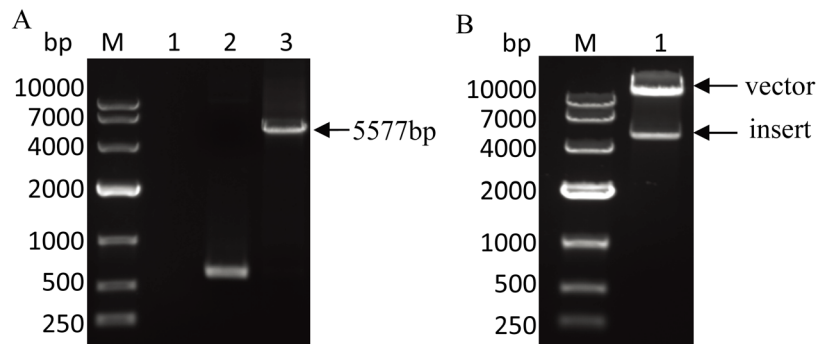

Fig. 4. Recombinant plasmid pD5H8-NS1 was identified by PCR amplification and restriction endonuclease digestion. (A) PCR amplification of the positive recombinant plasmid pD5H8-NS1 was performed and the size of the amplified fragment was 5,577 bp, while that of plasmid pD5H8 without inserts was 611 bp. Lane M, DL10000 DNA marker; lane 1, negative control; lane 2, pD5H8 vector fragment; lane 3, positive plasmid of pD5H8-NS1. (B) *Not*I restriction endonuclease digestion of the construct plasmid pD5H8-NS1 separated the vector fragment (12,899 bp) from the insert fragment (4,966 bp). Lane M, DL10000 DNA marker; lane 1, recombinant plasmid pD5H8-NS1 digested with *Not*I restriction endonuclease.

according to the sequence statistics calculated using the EditSeq software (Lasergene Genomics Suite, Madison, WI, U.S.A.). Comparison and analysis of the NS1 amino acid sequences of strain A/Duck/Fujian/FQ2/2007 (H9N2) with the vaccine strains A/Chicken/Beijing/1/94 (H9N2), A/Chicken/Fujian/G9/2009 (H9N2), A/Chicken/Shangdong/6/96 (H9N2), and other H9N2 subtype strains isolated in Fujian province [20], showed that the *NS1* gene is highly conserved in genetic evolution and the NS1 protein has high sequence identity (Fig. 3).

Construction of T. thermophila expression vector pD5H8-NS1

With the addition of the signal peptide to the 5′ terminal, and the His-tag to the 3′ terminal for purification, the total length of the NS1 nucleotide sequence was increased by 735 bp. PCR amplification of the recombinant plasmid pD5H8-NS1 was performed and the size of amplified fragment was 5,577 bp (Fig. 4A). After insertion into the pD5H8 vector, the recombinant plasmid pD5H8-NS1 was verified by restriction analysis. The vector fragment (12,899 bp) was separated from the insert expression cassette fragment (4,966 bp) (Fig. 4B).
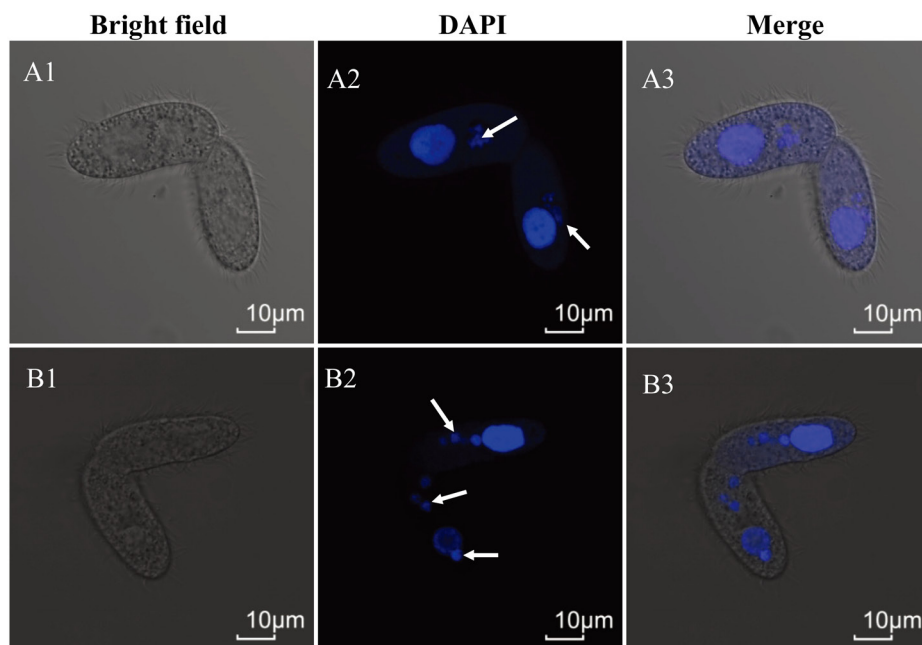
Fig. 5. Development of macronuclei and micronuclei in conjugating *Tetrahymena* cells. Mating *Tetrahymena* cells were observed under a laser scanning focus fluorescence microscope. The macronuclei and micronuclei were visualized with 4', 6-diamidino-2-phenylindole (DAPI) staining. Columns show bright field images (left column), DAPI staining (middle column), and merged images of bright field and DAPI staining (right column). The micronuclei are indicated by white hollow arrows. Scale bar=10 μm. The conjugating cells were pairing and two micronuclei in each cell could be observed after pairing for 4 hr (Fig. 5A). Four nuclei could be observed in each cell after pairing for 11 hr (Fig. 5B).

Construction and screening of transgenic T. thermophila strains

Starved cells began to pair at their anterior ends after mixing, followed by sexual reproduction (conjugation). The mating of cells and nuclei development were observed under a laser focus fluorescence microscope. The *T. thermophila* cilia were clearly visible. Two micronuclei in each cell could be observed after pairing for 4 hr (Fig. 5A). The zygotic nucleus divided twice mitotically, forming four nuclei that could be observed (Fig. 5B) in each cell after pairing for 11 hr. The pairing rate was more than 80%. Mating cells were then transformed by biolistic bombardment during anlagen formation of the macronucleus, indicating that homologous recombination was taking place between the donor strain (strong rDNA duplication function ability) and host strain (fast growth ability). Transformants were readily obtained following gradient selection by paromomycin and transgenic *T. thermophila* strains could be passaged stably.

Target protein expression and identification

Samples of disrupted cells from transgenic *T. thermophila* strains were separated into supernatant, proteinaceous gel, and white pellet fractions (Fig. 6A). A protein band of ~30 kDa was obtained from the gel sample (Fig. 6B). This showed that most of the target proteins were concentrated in the middle layer of lysates from recombinant *T. thermophila*. There was no target protein in the supernatant, and only a small amount of target protein was present in the precipitate. Western blotting was performed to detect the target NS1 protein, using specific positive rabbit antiserum. A visible band of 30 kDa was detected (Fig. 6C). To confirm the reliability of this result, the target protein band was analyzed by LC-MS/MS and found to match the NS1 protein of the A/Duck/Fujian/FQ2/2007 (H9N2) strain.

Development of indirect ELISA detection method

An indirect ELISA was established using the fusion protein as coating antigen. Our ELISA results indicated that the expressed protein had a specific reaction with rabbit antiserum, and the mean $OD_{492 \, nm}$ value was 1.125. Moreover, the expressed protein did not have any cross-reaction with anti-NDV, -IBDV, and -DTMUV or preimmunization rabbit serum (OD value: 0.107). Results depict the average of triplicate experiments (Fig. 7). These findings confirmed that the new NS1-ELISA method has high specificity and could be valuable for the development of diagnostic kits in the future.

DISCUSSION

Under selection pressure with paromomycin, the expression vector was genetically transformed using homologous recombination by replacing the rDNA of *Tetrahymena*. High-level transgene expression was then initiated by induction with cadmium. At present,
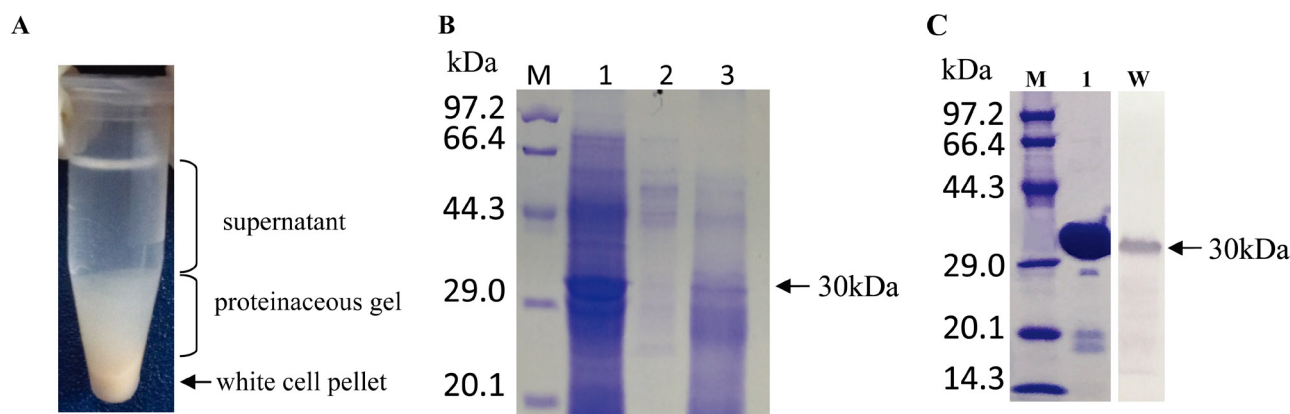
Fig. 6. SDS-PAGE and western-blot identification of NS1 protein. (A) The samples of disrupted cells from transgenic *Tetrahymena* strains were separated into supernatant, proteinaceous gel, and white pellet fractions after low speed centrifugation. (B) Samples of the supernatant, proteinaceous gel, and precipitate were loaded onto a 12% SDS-PAGE under reducing conditions. Lane M, molecular weight marker; lane 1, protein proteinaceous gel; lane 2, supernatant; lane 3, precipitate. (C) Lane M, molecular weight marker; lane 1, purified recombinant protein; lane W, protein detected by immunoblotting with rabbit antiserum against strain A/Duck/Fujian/FQ2/2007 (H9N2).

the methods for transformation of *T. thermophila* include electroporation and particle bombardment [5, 8]. This study applied the simple and efficient biolistic bombardment to transform the embedded DNA into *T. thermophila*, causing less cellular damage [5]. We integrated foreign genes by homologous recombination to produce stable gene micronucleus transformants, and finally obtained transgenic *T. thermophila* strains that expressed NS1 protein with high efficiency.

Available expression systems mainly include *E. coli*, animal cell, baculovirus expression systems, and yeast cell systems. The limitations of *E. coli* systems include the lack of transcriptional or post-translational modification apparatus. Animal cell and baculovirus expression systems present expressed proteins with natural biological activity and function; however, these systems are not ideal for large-scale culture due to their complex handling and high cost. Yeast cell expression systems are easy to handle and more cost-effective, but it is difficult to disrupt yeast cell walls, which greatly impedes downstream purification. The main advantage of the *Tetrahymena* expression system is that it can achieve

Fig. 7. Specificity of indirect ELISA using the expressed protein as coating antigen. Except for the rabbit antiserum against A/Duck/Fujian/FQ2/2007 (H9N2), the expressed protein did not cross-react with anti-NDV, -IBDV, and -DTMUV or preimmunization rabbit serum. FQ2, rabbit antiserum against A/Duck/Fujian/FQ2/2007; NC, preimmunization rabbit serum.

high-level expression of recombinant proteins with better antigenicity and natural function [1, 9]. Moreover, post-translational modifications of recombinant proteins, including disulfide bridge formation, glycosylation, acylation, and phosphorylation can be processed similarly to those in mammalian cells [1]. Therefore, the *Tetrahymena* system is ideal for the expression of biologically active proteins. Gaertig *et al.* reported that a parasite antigen was expressed on the cell surface of transformed *Tetrahymena* and used for the development of a vaccine against *Ichthyophthirius multifiliis* [7]. Several genes have been efficiently expressed in *Tetrahymena*, including the gene encoding human DNaseI and genes from human pathogens, such as *Plasmodium falciparum*, *Mycoplasma*, and *Leishmania* [2, 7, 18].

One limitation of the *Tetrahymena* expression system is codon bias necessitating codon optimization for the genes of all expressed proteins before use. Mochizuki [12] demonstrated that the codon-optimized neo gene cassette resulted in a ten-fold increase of drug-resistant transformants compared with the non-codon-optimized original neo gene. The study also emphasized the importance of codon optimization for transgene expression in *Tetrahymena*. A non-optimized codon in *Tetrahymena* might strongly affect expression efficiency. Furthermore, only a single stop codon (UGA) is recognized as a translation terminator in *Tetrahymena*, while the other two commonly used stop codons, UAA and UAG, encode for glutamine.

The clinical application of NS1 protein mainly focuses on the prevention and diagnosis of avian influenza. The NS1 protein can stimulate infected hosts to elicit an immune response and produce corresponding antibodies. So far, ELISA, agar gel precipitin, and hemagglutination inhibition tests are considered the international standard detection methods for the serodiagnosis of avian influenza
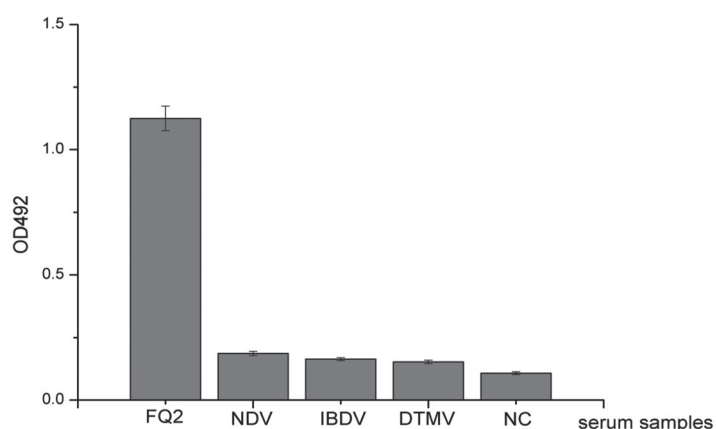
in poultry. However, these assays cannot differentiate between infected and immunized poultry when commercial inactivated avian influenza vaccines are used, which interferes with serologic surveillance and hinders the prevention and control of avian influenza. Thus, it is imperative to develop a more sensitive diagnostic system that can differentiate infected from vaccinated poultry. The NS1 protein is remarkably conserved. More importantly, the NS1 protein is a non-structural protein that only exists in virus-infected host cells but not in virions. These properties make NS1 protein an ideal diagnostic marker of early infection that can differentiate infected from vaccinated poultry. A previous study demonstrated that antibodies against NS1 could be detected in the sera of horses experimentally infected with the H3 subtype of influenza virus but not in the sera of horses immunized with inactivated viruses [13]. Furthermore, Tumpey *et al.* [17] clinically distinguished between infected and vaccinated birds using expressed recombinant NS1 proteins as coating antigens. However, the above studies utilized *E. coli* systems to express the NS1 protein [19], wherein the expressed protein was isolated in the form of inclusion bodies. After laboratory manipulations, such as denaturation and renaturation, some proteins fold incorrectly, possibly exposing internal antigenic epitopes. If these proteins are used as coating antigens, it may cause false-positive results during clinical serologic testing. Using the *Tetrahymena* system, the expressed proteins were able to fold correctly, retain conformation-dependent epitopes, and achieve better antigenicity. In our study, the expressed protein only reacted with rabbit antiserum and did not cross-react with anti-NDV, -IBDV, -DTMUV, or preimmunization rabbit serum, indicating that preliminary NS1-ELISA method has good specificity. The thirty sera samples being tested were from large-scale domestic duck farms, in which the ducks were immunized with inactivated vaccines, and the serum test results were negative (data not shown). More clinical sera samples need to be collected from domestic poultry farms and live poultry markets for further research. Using the expressed proteins in this study may increase the potential to develop effective diagnostic kits to differentiate infected from vaccinated poultry in the near future.

Previous studies have shown that *Tetrahymena* can be cultured in large-scale bioreactor fermentation systems at concentrations as high as 2.2×10^7 cells/ml, which enables production of multiple NS1 protein antigens to develop ELISA diagnostic kits or manufacture influenza vaccines. AIV are highly variable and currently available vaccines are often unable to cope with the latest outbreaks. *Tetrahymena* is a novel alternative expression system due to its rapid cell growth to high densities and it can be developed to produce viral proteins that can be utilized for subunit vaccine production during influenza pandemics.

In summary, the AIV NS1 protein was successfully expressed in *T. thermophila*, establishing a good foundation for further studies on the development of heterologous genes. This emerging technology might provide new ideas and directions for the prevention and control of future influenza outbreaks.

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