Research Note—

New Approach to Delist Highly Pathogenic Avian Influenza Viruses from BSL3+ Select Agents to BSL2 Non-Select Status for Diagnostics and Vaccines

Samadhan J. Jadhao and David L. Suarez

Southeast Poultry Research Laboratory, U.S. Department of Agriculture, Agricultural Research Service, 934 College Station Road, Athens, GA 30605

Received 18 May 2009; Accepted and published ahead of print 6 October 2009

SUMMARY. Highly pathogenic avian influenza viruses (AIVs) are Select Agents in the United States and are required to be handled in bio-containment level-3 enhanced (BSL3+) facilities. Using a reverse genetics system, we attenuated a highly pathogenic virus, with the goal of making it low pathogenic and having it delisted as a Select Agent so that it could be handled in a bio-containment level-2 facility for diagnostic or vaccine production applications. We utilized two approaches to attenuate the target AIV by mutating the highly pathogenic hemagglutinin (HA) cleavage site to be low pathogenic and by replacing the full-length NS gene segment with a naturally truncated 124-amino acid NS1 coding gene from A/turkey/Oregon/73 (H7N3) virus (tkOR71 trNS1). To delist an AIV so that it can be handled in a BSL2 facility, the amino acid sequence of the HA cleavage site of the rescued virus must be confirmed to be compatible with a low-pathogenic AIV; it should not plaque in cell culture without supplementation of exogenous trypsin; and intravenous pathotyping in 4–6-wk-old specific-pathogen-free chickens must confirm that the virus is low pathogenic. The candidate A/duck/Vietnam/Bagieu/09/07 (rH5N1/PR8/trNS1) virus with five PR8 internal genes, tkOR71 trNS1 gene, and A/chicken/Indonesia/7/03 N1 neuraminidase gene was constructed. The virus was shown to not plaque in cell culture without addition of trypsin; and intravenous pathotyping in 4–6-wk-old specific-pathogen-free chickens confirmed that the virus was non-pathogenic. The candidate A/chicken/Indonesia/7/03 N1 neuraminidase gene was constructed. The virus was shown to not plaque in cell culture without addition of trypsin. The virus was low pathogenic in the standard intravenous pathotyping test (IVPI = 0) and also caused no disease in a separate intranasal inoculation test in 4-wk-old specific-pathogen-free chickens, thus demonstrating that the virus is suitable for de-selection.

RESUMEN. Nota de Investigación—Enfoque nuevo para la eliminación de un virus de influenza aviar de alta patogenicidad de la lista de agentes selectos BSL3+ para ser incluido en el estatus no selecto BSL2 con fines de diagnóstico y elaboración de vacunas.

Los virus de alta patogenicidad de influenza aviar (AIV, por sus siglas en inglés) son Agentes Infecciosos Selectos en los Estados Unidos y requieren para su manejo de instalaciones con un nivel de biocontención tipo 3 extra (BSL3+, por sus siglas en inglés). Usando un sistema de reversión genética, se atenuó un virus de alta patogenicidad con el objetivo de hacerlo de baja patogenicidad y lograr que se elimine de la lista de agentes selectos para de esta manera, poder manejarlo con fines diagnósticos o para la producción de vacunas en instalaciones de un nivel de biocontención 2 (BSL2, por sus siglas en inglés). Se utilizaron dos enfoques para la atenuación del virus seleccionado, mediante mutación en el sitio de disociación de la hemaglutinina (HA, por sus siglas en inglés) de alta patogenicidad, para convertirlo en un virus de baja patogenicidad, o por el reemplazo del segmento completo del gene NS por un gene truncado de manera natural que codifica para una proteína NS1 de 124 aminoaídos y que pertenece al virus A/pavo/Oregon/73 (H7N3), (gene tkOR71 trNS1). Para retirar de la lista de agentes selectos a un virus de influenza aviar y de esta manera poder manejarlo en instalaciones tipo BSL2, la secuencia de aminoaídos del sitio de disociación de la hemaglutinina del virus rescatado debe ser confirmada como compatible con un virus de influenza aviar de baja patogenicidad; éste no debe formar placas en cultivo celular sin la suplementación de trypsin exógena y la tipificación de la patogenicidad mediante inoculación intranasal en aves libres de patógenos específicos de 4 a 6 semanas debe confirmar que el virus es de baja patogenicidad. Se construyó un virus candidato, el virus A/pavo/Vietnam/Bagieu/09/07 (rH5N1/PR8/trNS1) con cinco genes internos de PR8, con el gene tkOR71 trNS1, y el gene de la neuraminidasa N1 del virus A/pollo/Indonesia/7/03. El virus no produjo placas en los cultivos celulares sin la adición de trypsin. El virus se comportó como un virus de baja patogenicidad en la prueba estándar de tipificación de la patogenicidad por inoculación intranasal mostrando un índice de patogenicidad intranasal (IVPI, por sus siglas en inglés) de cero y no causó enfermedad después de la inoculación intranasal en pollos libres de patógenos específicos de cuatro semanas de edad, lo que demostró que el virus era apto para ser deleccionado.

Key words: avian influenza virus, reverse genetics, NS1 gene, avian influenza vaccine, select agents

Abbreviations: AIV = avian influenza virus; bp = base pair; BSL3+ = bio-containment level-3; ECE = embryonated chicken eggs; EID<sub>50</sub> = 50% egg infectivity; HA = hemagglutinin; MDCK = Madin Darby Canine Kidney; PBS = phosphate-buffered saline; RT-PCR = reverse transcriptase-PCR; SPF = specific pathogen free

Highly pathogenic H5N1 avian influenza viruses (AIV) have become endemic in poultry in several Asian and at least one African country, resulting in unprecedented economic loss to poultry farmers and zoonotic infections in people, leading to fatal illnesses (1,2,3,5,7,9,10,11,12,16,20,22,23,27,28,29). Under these circumstances, control and prevention of avian influenza in poultry is a high priority. In addition to the traditional culling approach, preventive vaccination for avian influenza in poultry has gained importance because it has yielded some success in controlling the disease and because of the increasing acceptance of vaccination as a control tool in some affected Asian countries. The success of preventive vaccination programs depends in part on the availability of vaccine strains that are antigenically matched to the circulating highly

---

*Corresponding author. E-mail: david.suarez@ars.usda.gov*
pathogenic AIV field strains (14,25). Because naturally circulating low pathogenic AIVs provide a poor antigenic match against the circulating Asian H5N1 lineage of viruses, the use of plasmid-based reverse genetics and site-directed mutagenesis has become indispensable as a way to produce antigenically matched vaccine strains. A vaccine seed strain can be attenuated by mutating the hemagglutinin (HA) cleavage site so that it has a low pathogenicity cleavage site, and this approach allows the virus to remain antigenically matched to the parent strain, because the cleavage site is distantly located in relation to neutralizing sites on the HA protein (17,24). Alternative methods of attenuation have been described, including changes to the NS1 protein. One study characterized a variant of A/turkey/Oregon/71 (H7N3) that had a naturally truncated 124–amino acid NS1 protein, compared with the usual 230–amino acid NS1 protein. The variant virus with the truncated NS1 protein did not efficiently block the induction of type I interferon, which the parent virus with the full-length NS1 gene did, and it exhibited an attenuated pathotype in chickens and poorly transmitted to in-contact sentinel chickens (6,26).

Highly pathogenic AIVs are considered Select Agents in the United States, and the live virus is required to be handled in an enhanced biocontainment level-3 (BSL-3+) facility. Although an HPAI virus can be attenuated to be low pathogenic using reverse genetics, these viruses are still considered Select Agents and must be handled under BSL-3 conditions, which limits how they can be used. A virus can be delisted as a Select Agent in the United States if it meets certain requirements. In brief, the HA cleavage site must be changed so that it is compatible with a low pathogenic virus; it must be shown that the virus can no longer grow in cell culture without supplementation of exogenous trypsin in cell culture medium; and, finally, the virus must be tested in the standard intravenous pathotyping test in chickens, per World Organization for Animal Health guidelines. If a virus meets all of these conditions, it may be considered for deselection. In addition to the change at the HA cleavage, we considered it beneficial to include additional attenuation factors in the virus to increase the safety of working with live virus in BSL-2 facilities. The use of a truncated NS1 gene of A/turkey/Oregon/71 (H7N3) was selected as a way to attenuate the virus in animals; this selection will still allow the virus to be grown to high titer in 9- to 10-day-old embryonating chicken eggs. Based on this hypothesis, we used a reverse genetics approach to incorporate both changes: the H5 HA cleavage site mutation and a truncated NS1 protein in the recombinant H5N1 virus. Further, we evaluated the rescued virus for its ability to replicate in chickens in an attempt to have the virus deselacted.

**MATERIALS AND METHODS**

**Reverse transcriptase-PCR (RT-PCR) and nucleotide sequencing.** The AIVs used in this study were obtained from the Southeast Poultry Research Laboratory (USDA-ARS, Athens, GA). The H5 HA gene of A/duck/Vietnam/Bacgieu/09/07 (H5N1) virus was reverse transcribed into complementary DNA using influenza A virus 12 mer primer AGGAAAGACAGG and AMV reverse transcription system (Promega, Inc., Madison, WI). Full-length H5 HA was PCR amplified using BsmBI HA forward 5'-TATTCGTCCTCAGGGAGCAGAACGAGG-3' and BsmBI HA reverse 5'-TATTCGTCCTCAGGGAGCTAGTA-GAAAACGGGTTT-3' primers and PfuUltra® II Fusion HS DNA Polymerase (Stratagene, Inc., Cedar Creek, TX). The H5 HA gene amplicon was restriction digested with BsmBI enzyme and cloned into a bi-directional 425–base pair (bp) promoter chicken RNA polymerase I reverse genetics vector (Jadhao and Suarez, unpubl. data). Automated nucleotide sequencing of the H5 HA gene was performed using gene-specific primers and a PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (PerkinElmer, Foster City, CA) on a 3730 PerkinElmer machine. Using site-directed mutagenesis PCR, the highly pathogenic H5 HA cleavage sequence PQREGRRK/GLF was mutated to low pathogenic PQRETR/GLF cleavage site. The mutated H5 HA cleavage site sequence was also confirmed by nucleotide sequencing. The AIV A/turkey/Oregon/71 (H7N3), with a truncated NS1 protein of only 124 amino acids, was used as the sequence to clone the NS gene. As was the case with the H5 HA gene, the NS gene segment of A/turkey/Oregon/71 (H7N3) virus variant was amplified using primers BsmBI NS forward 5'-TATTCTGCTCAGGGAGCAGAAGGGGT-3' and BsmBI NS reverse 5'-TATTCTGCTCAGGGAGCAGAAGGGGT-3' cloned in the reverse gene vector. The NS segment was sequenced to confirm the truncated NS1 124–amino acid length. The NS gene segment-specific primers were used to perform one-step RT-PCR to demonstrate truncated NS segment in the recombinant H5N1 (rH5N1/PR8/trNS1) virus.

**Generation of recombinant AIV using reverse genetics.** The chicken RNA polymerase I 425 promoter-based bi-directional plasmid-based reverse genetics system was constructed for A/Puerto Rico/8/34 (H1N1) strain (Jadhao and Suarez, unpubl. data), similar to the 250–bp chicken RNA polymerase I promoter-based bi-directional plasmid-based reverse genetics system described previously by Massin et al. (18). To generate a recombinant virus, the mutated low pathogenic RETR cleavage site coding H5 subtype HA gene was derived from A/duck/Vietnam/Bacgieu/09/07 (H5N1) virus, the N1 subtype neuraminidase gene was from A/chicken/Indonesia/7/03 (H5N1) virus, and the internal genes (PB2, PB1, PA, NP, and M), with the exception of the NS gene, originated from the A/Puerto Rico/8/34 (H1N1) strain. The NS gene segment was derived from the A/turkey/Oregon/71 (H7N3) virus variant coding a truncated 124–amino acid NS1 protein. The DF-1 cell line, procured from American Type Culture Collection and maintained at Southeast Poultry Research Laboratory (USDA-ARS, Athens, GA), was used for transfection. The recombinant H5N1/PR8/trNS1 AIV was rescued in chicken fibroblast (DF-1) cells using an 8 bi-directional chicken RNA polymerase I promoter-driven plasmid-based reverse genetics system. The transfection procedure was similar to previously described protocols (18,19), but with a few modifications. Briefly, 1 μg each of the bi-directional plasmid and 16 μl of Lipofectamine 2000 (Invitrogen, Inc., Carlsbad, CA) was used to transfect a six-well plate dish of chicken fibroblast (DF-1) cells. After incubation for 72 hr in 37 C/5% CO2 incubator, the transfection supernatants were inoculated in 9-day-old embryonated specific-pathogen-free (SPF) chicken eggs. The allantoic fluids were harvested after 3–4 days from the eggs and tested for virus rescue by hemagglutination and egg infectivity assay (13). The sterility of the allantoic fluid with HA-positive test was ascertained by inoculation of thioglycolate broth tubes at 37 C for 1 wk. The stock virus was produced by a second limiting dilution passage in the SPF embryonated chicken eggs (ECE).

**Confirmation of low pathogenic HA cleavage site and truncated NS1 gene.** The H5 HA and NS gene segment of the reverse genetics rescued H5N1 virus was amplified using gene-specific primers (primer sequence available upon request) in a one-step RT-PCR (Qiagen, Inc., Valencia, CA). The H5 HA cleavage site low pathogenic RETR sequence was confirmed by direct nucleotide sequencing, as described earlier in this paper. The truncated NS segment of 700-bp size was confirmed by 1% agarose gel electrophoresis.

**Growth properties in Madin Darby Canine Kidney (MDCK) cell culture.** The plaque-forming ability of the recombinant A/duck/Vietnam/Bacgieu/09/07 (rH5N1/PR8/trNS1) virus strain with mutated low pathogenic RETR cleavage site sequence and truncated NS1 gene from A/turkey/Oregon/71 (H7N3) virus variant was tested in MDCK cell line in the presence and absence of exogenous i-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin. The confluent MDCK cell cultures in the six-well culture plates were infected with 10-fold dilutions of the virus in Dulbecco’s Minimum Essential Medium at 37 C/5% CO2 atmosphere. After 1 hr, medium was removed and overlaid with 0.9% agar prepared in phenol red free
MEM medium with or without 1 μg/ml TPCK-treated trypsin. The plates were incubated at 37°C/5% CO₂ atmosphere for 4 days and fixed with 1% neutral formalin for at least 24 hr. Following removal of agar, the fixed cell cultures were stained with 0.05% neutral red to better visualize the plaques.

**Growth properties in ECE.** The recombinant A/duck/Vietnam/Bacleiu/09/07 (rH5N1/PR8/trNS1) virus was diluted 10-fold in phosphate-buffered saline (PBS) with 1X antibiotic and antimycotic solution and inoculated at 0.1 ml via chorio-allantoic cavity route in 9-day-old SPF-ECE (n = 5/virus dilution) and incubated for 3–4 days. The allantoic fluids were harvested from the chilled eggs and tested by HA assay using 0.5% (v/v) chicken erythrocytes. Fifty percent egg infectivity (EID₅₀/ml) virus titers were determined by the Reed and Muench method (21).

**Pathogenicity of the recombinant virus in chickens.** The animal experiments were conducted with the approval and oversight of the Southeast Poultry Research Laboratory Institutional Animal Care and Use Committee. Eight 4-wk-old white leghorn SPF chickens were inoculated by intravenous route or intranasal route with 0.2 ml of 1:10 dilution of the A/duck/Vietnam/Bacleiu/09/07 (rH5N1/PR8/trNS1) virus strain prepared in PBS (pH 7.2). The birds were housed in negative-pressure Horsfal units fitted with HEPA filters in an enhanced BSL-3 animal building. Water and feed were provided to the birds ad libitum. The chickens were monitored for 10 days for the presence of clinical signs or mortality, as required for AIV standard pathotyping. Oro-pharyngeal swabs were collected in 1-ml PBS/antibiotic-antimycotic solution (pH 7.2) on day 2 and day 4 postchallenge. RNA was extracted from the oro-pharyngeal swabs using the Trizol and magnetic bead–based extraction protocol (8). Influenza A virus matrix gene-specific real-time RT-PCR was performed to assess the extent of the oro-pharyngeal virus shedding (15). A/duck/Vietnam/Bacleiu/09/07 (rH5N1/PR8/trNS1) virus aliquot with predetermined EID₅₀ titer was used for RNA extraction and was included to generate a standard curve to determine the virus shedding in the oro-pharyngeal swabs of chickens in the pathotyping experiment (15). To assess the seroconversion, serum samples were harvested on day 14 postchallenge, and an agar gel precipitation test was performed using reagents procured from the National Veterinary Services Laboratories in Ames, IA (4).

**RESULTS AND DISCUSSION**

The goal of this work was to produce an attenuated virus from a highly pathogenic H5N1 virus using a protocol that would potentially allow the virus to be desected so that it could be safely handled under BSL-2 conditions. Using a chicken RNA polymerase I 425-bp promoter plasmid-based reverse genetics system, a virus was rescued that contained an H5 HA gene from A/duck/Vietnam/Bacleiu/09/07 that had a mutated cleavage site characteristic of a low pathogenic RETR sequence, a truncated NS1 gene from A/turkey/Oregon/71 (H7N3) virus. The recombinant A/duck/Vietnam/Bacleiu/09/07 (rH5N1/PR8/trNS1) virus was diluted 10-fold in phosphate-buffered saline (PBS) with 1X antibiotic and antimycotic solution and inoculated at 0.1 ml via chorio-allantoic cavity route in 9-day-old SPF-ECE (n = 5/virus dilution) and incubated for 3–4 days. The allantoic fluids were harvested from the chilled eggs and tested by HA assay using 0.5% (v/v) chicken erythrocytes. Fifty percent egg infectivity (EID₅₀/ml) virus titers were determined by the Reed and Muench method (21).

**Pathogenicity of the recombinant virus in chickens.** The animal experiments were conducted with the approval and oversight of the Southeast Poultry Research Laboratory Institutional Animal Care and Use Committee. Eight 4-wk-old white leghorn SPF chickens were inoculated by intravenous route or intranasal route with 0.2 ml of 1:10 dilution of the A/duck/Vietnam/Bacleiu/09/07 (rH5N1/PR8/trNS1) virus strain prepared in PBS (pH 7.2). The birds were housed in negative-pressure Horsfal units fitted with HEPA filters in an enhanced BSL-3 animal building. Water and feed were provided to the birds ad libitum. The chickens were monitored for 10 days for the presence of clinical signs or mortality, as required for AIV standard pathotyping. Oro-pharyngeal swabs were collected in 1-ml PBS/antibiotic-antimycotic solution (pH 7.2) on day 2 and day 4 postchallenge. RNA was extracted from the oro-pharyngeal swabs using the Trizol and magnetic bead–based extraction protocol (8). Influenza A virus matrix gene-specific real-time RT-PCR was performed to assess the extent of the oro-pharyngeal virus shedding (15). A/duck/Vietnam/Bacleiu/09/07 (rH5N1/PR8/trNS1) virus aliquot with predetermined EID₅₀ titer was used for RNA extraction and was included to generate a standard curve to determine the virus shedding in the oro-pharyngeal swabs of chickens in the pathotyping experiment (15). To assess the seroconversion, serum samples were harvested on day 14 postchallenge, and an agar gel precipitation test was performed using reagents procured from the National Veterinary Services Laboratories in Ames, IA (4).

**RESULTS AND DISCUSSION**

The goal of this work was to produce an attenuated virus from a highly pathogenic H5N1 virus using a protocol that would potentially allow the virus to be desected so that it could be safely handled under BSL-2 conditions. Using a chicken RNA polymerase I 425-bp promoter plasmid-based reverse genetics system, a virus was rescued that contained an H5 HA gene from A/duck/Vietnam/Bacleiu/09/07 that had a mutated cleavage site characteristic of a low pathogenic RETR sequence, a truncated NS1 gene from A/turkey/Oregon/71 (H7N3) virus. The chickens were monitored for 10 days for the presence of clinical signs or mortality, as required for AIV standard pathotyping. Oro-pharyngeal swabs were collected in 1-ml PBS/antibiotic-antimycotic solution (pH 7.2) on day 2 and day 4 postchallenge. RNA was extracted from the oro-pharyngeal swabs using the Trizol and magnetic bead–based extraction protocol (8). Influenza A virus matrix gene-specific real-time RT-PCR was performed to assess the extent of the oro-pharyngeal virus shedding (15). A/duck/Vietnam/Bacleiu/09/07 (rH5N1/PR8/trNS1) virus aliquot with predetermined EID₅₀ titer was used for RNA extraction and was included to generate a standard curve to determine the virus shedding in the oro-pharyngeal swabs of chickens in the pathotyping experiment (15). To assess the seroconversion, serum samples were harvested on day 14 postchallenge, and an agar gel precipitation test was performed using reagents procured from the National Veterinary Services Laboratories in Ames, IA (4).
Oregon/71 (H7N3) virus variant, the N1 gene from chicken/Indonesia/07/03, and the remaining genes (PB2, PB1, PA, NP, and M) from A/Puerto Rico/34 (H1N1) strain in the gene constellation. To clearly identify the recombinant virus, a list of the origins of all gene segments was included in the virus name, viz. A/duck/Vietnam/Baclieu/09/07 H5 HA × A/chicken/Indonesia/703 N1 NA × A/ Puerto Rico/8/34 MA, NP, PA, PB1, PB2 × A/turkey/Oregon/71 NS truncated.

The recombinant A/duck/Vietnam/Baclieu/09/07 (rH5N1/PR8/trNS1) virus H5 HA gene was confirmed to have a low pathogenic cleavage site of RETR/GLF (Fig. 1a) by sequencing. The RT-PCR test using NS segment-specific primers indicated the presence of a truncated NS segment of approximately 700 bp (Fig. 1b). The recombinant virus did not form plaques in MDCK cell culture in the absence of exogenous supplementation of TPCK-treated trypsin (Fig. 1c). However, the same virus formed clear plaques in MDCK cells in 4 days when the agar overlay was supplemented with exogenous TPCK-treated trypsin (Fig. 1c). The growth property of A/duck/Vietnam/Baclieu/09/07 (rH5N1/PR8/trNS1) virus in 9-day-old ECE indicated that it grows to infectivity titer of 10⁶ EID₅₀/ml and HA titers as high as 512. Negative testing of the recombinant A/duck/Vietnam/Baclieu/09/07 (rH5N1/PR8/trNS1) virus RNA by real-time RT-PCR for Newcastle disease virus ensured that the viral aliquots were free of this viral pathogen.

The intravenous pathotyping test, required for deselection of the virus, and the intranasal infectivity test indicated that the virus did not cause clinical signs over a period of 14 days in inoculated chickens. No sero-conversion was found by the influenza A-virus-specific agar gel immunodiffusion assay both in intravenous and intranasally inoculated chickens. The influenza A virus matrix gene real-time RT-PCR test on the oro-pharyngeal swabs did not detect viral shedding on days 2 or 4 postchallenge. The agar gel immunodiffusion assay and negative viral shedding data indicate that the recombinant A/duck/Vietnam/Baclieu/09/07 (rH5N1/PR8/trNS1) virus either did not replicate or replicated only poorly in chickens and would be a safer alternative from which to produce diagnostic antigens for handling in the laboratories using BSL-2 containment levels.

The data from this rescued virus demonstrate that the virus is attenuated and meets the standards for being deselectable. Regulatory approval is still required before a virus can be deselectated. Upon U.S. Department of Agriculture approval, the mutated nonpathogenic recombinant H5N1 viruses would be transferred to the BSL-2 laboratory for further work. This study describes a protocol to generate more attenuated exotic rH5N1/PR8/trNS1 AIVs to handle in the laboratories with lower or BSL-2 containment levels.

REFERENCES


ACKNOWLEDGMENTS

We thank Dr. Ruben Donis (Centers for Disease Control and Prevention, Atlanta, GA) for providing the MDCK cell line. The excellent technical assistance of Aniko Zsak, Suzanne DeBlois, and Joan Beck is gratefully acknowledged for this ongoing research project. This research project is being supported by U.S. Department of Agriculture grant 901-6612-480, and there is an Interagency agreement with the Centers for Disease Control and Prevention (908-6612-820).