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H7N3 Avian Influenza Virus Found in a South American Wild Duck Is Related to the Chilean 2002 Poultry Outbreak, Contains Genes from Equine and North American Wild Bird Lineages, and Is Adapted to Domestic Turkeys

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An H7N3 avian influenza virus (AIV) was isolated from a Cinnamon Teal (*Anas cyanoptera*) (A/CinnamonTeal/Bolivia/4537/01) during a survey of wild waterfowl in Bolivia in 2001. The NA and M genes had the greatest identity with North American wild bird isolates, the NS was most closely related to an equine virus, and the remaining genes were most closely related to isolates from an outbreak of H7N3 in commercial poultry in Chile in 2002. The HA protein cleavage site and the results of pathogenesis studies in chickens were consistent with a low-pathogenicity virus, and the infective dose was 10^5 times higher for chickens than turkeys.

Prior to an outbreak of avian influenza virus (AIV) in commercial chickens and turkeys in Chile in 2002 (7), the detection of AIV had not been reported in South America. Importantly, commercial poultry is routinely monitored for AIV to comply with export regulations; however, surveillance of wild aquatic birds for AIV in South America has historically been minimal. Prior to the outbreak in Chile, a study was conducted in Bolivia in 2001. A total of 93 samples (24 from Cinnamon Teal) were collected from 11 species, including ducks, sheldgeese, and doves. One AIV was isolated from a Cinnamon Teal (*Anas cyanoptera*), collected on 27 October 2001 (UAM 19,003: Bolivia, Dpto. La Paz, Lake Titicaca [16°11′45″S, 68°37′28″W]; elevation, 3,808 m). This isolate represents the first report and earliest isolate of an AIV from a wild bird in South America. Here we evaluate the genetics and pathobiology of this isolate.

Sample collection and initial screening for AIV. Cloacal swabs were collected from wild waterfowl, placed in brain heart infusion broth, frozen in liquid nitrogen, and shipped frozen to SEPRL for processing (samples were not processed immediately for logistical reasons). RNA was extracted from cloacal swab material with TRIzol LS reagent (Invitrogen, Inc., Carlsbad, CA) in accordance with the manufacturer's instructions. RNA was tested for AIV by real-time reverse transcription-PCR (rRT-PCR) directed to the matrix (M) gene, which detects all type A influenza viruses as previously reported (4). Virus isolation was performed in embryonated chicken eggs by using standard procedures (9) with swab material from rRT-PCR-positive samples.

Genetic analysis. The entire coding sequences of all eight viral gene segments were amplified by RT-PCR as previously reported (6) and directly sequenced with the BigDye terminator kit (Applied Biosystems, Foster City, CA) on an ABI 3730 (Applied Biosystems, Foster City, CA). The GenBank accession numbers are DQ525411 through DQ525418. BLAST analysis was used to initially identify the most closely related type A influenza viruses. Once the lineage was defined, all available isolates for each gene from the most closely related lineage were included in a multiple alignment by CLUSTAL V, and the percent identities were calculated.

Phylogenetic analysis was performed with phylogenetically representative isolates from all available sequences for each gene. Multiple alignments for each gene were performed with CLUSTAL V (Lasergene V.6; DNAStar, Madison, WI). Phylogenetic trees were generated using maximum-parsimony by a heuristic search with 500 bootstrap replicates (PAUP*4.0b10; Sinauer Associates, Sunderland, MA). The HA tree was rooted with Fowl plague virus/Rostock/34, and the NS tree was rooted with the influenza virus type B isolate B/Lee/40.

This virus isolate, A/Cinnamon Teal/Bolivia/4537/01, was

TABLE 1. Type A influenza virus isolates with the highest nucleotide identity to A/CinnamonTeal/Bolivia/4537/01 by individual gene segment

Gene segment (abbreviation)	Subtype	Isolate with highest sequence identity	% Identity
Hemagglutinin (HA)	H7	Chicken/Chile/176822/02	96.6
Neuraminidase (NA)	N3	Emu/TX/25414-1/95	96.7
Matrix (M)		Blue-winged Teal/LA/B182/86	97.4
Nonstructural (NS)	A	Equine/LaPlata/1/88	93.6
Nucleoprotein (NP)		Chicken/Chile/176822/02	95.8
Polymerase acid protein (PA)		Chicken/Chile/176822/02	90.1
Polymerase basic protein 1 (PB1)		Chicken/Chile/176822/02	95.2
Polymerase basic protein 2 (PB2)		Chicken/Chile/176822/02	95.5

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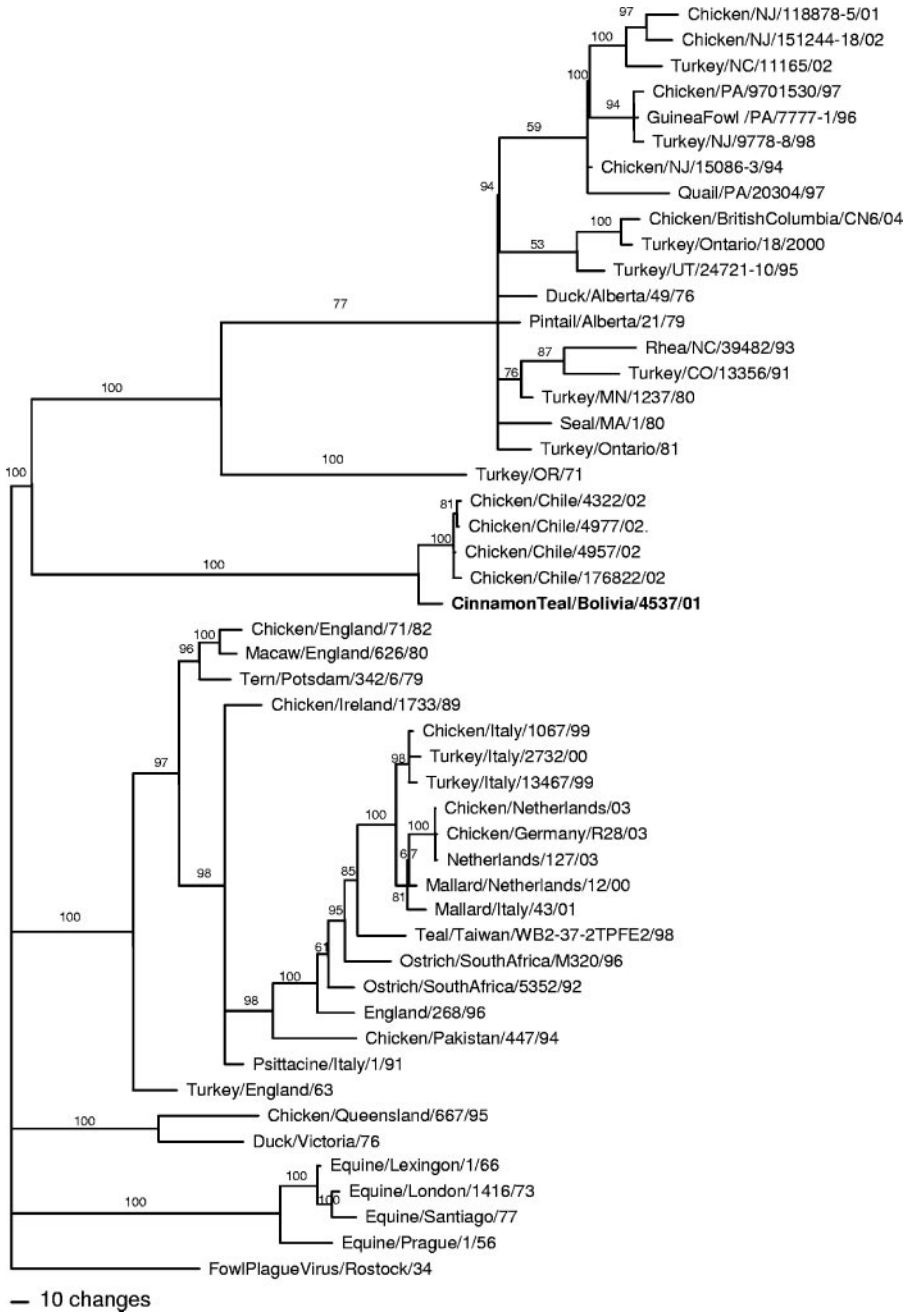

FIG. 1. Phylogenetic tree of the H7 subtype hemagglutinin gene. The tree was constructed with PAUP* 4.0b10 (Sinauer Associates, Sunderland, MA) using maximum parsimony, heuristic search, and 500 bootstrap replicates (bootstrap values are shown on tree) and rooted with Fowl plague virus/Rostock/34. States are abbreviated by their standard two-letter postal codes.

determined to be the H7 HA, N3 NA, and NS type A subtypes by gene sequencing (Table 1). Based on phylogenetic analysis, the HA (Fig. 1), NP, PA, PB1, and PB2 genes were most closely related to the AIV isolates collected from chickens and turkeys during an outbreak in commercial poultry in Chile in 2002. The HA protein cleavage site was consistent with a low-pathogenicity AIV (LPAIV) and identical to the cleavage site of the LPAIV from commercial poultry in Chile in 2002.

The NA and M genes were most closely related to genes from North American wild bird lineages. The NA had 96.7% nucleotide (nt) identity with A/Emu/TX/25412-1/95, and the M gene had 97.4% nt identity with A/BlueWingedTeal/LA/B182/86 (Table 1). The NS gene was most closely related to the NS gene of an equine virus (Fig. 2), with a 93.6% nt identity to A/Equine/LaPlata(Argentina)/1/88, although some North American wild bird genes had as much as 90 to 92% identity.

Analysis of deduced protein sequences reveals trends similar to what is seen in the nucleotide sequence analysis. In addition, the general protein sequence features of this virus are consistent with a wild-bird origin virus; residue 627 in PB2 is glutamic

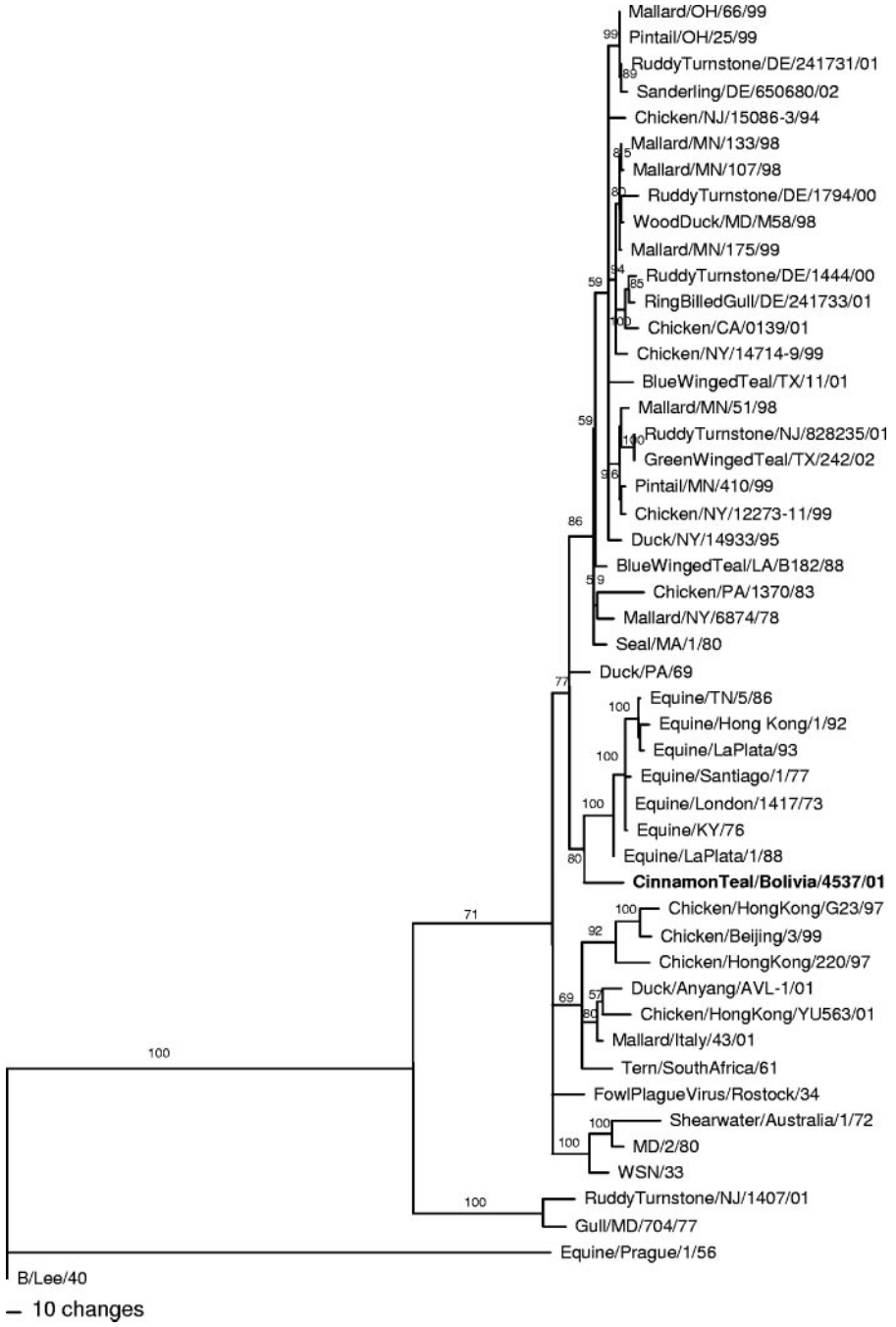

FIG. 2. Phylogenetic tree of the subtype A nonstructural gene. The tree was constructed with PAUP* 4.0b10 (Sinauer Associates) using maximum parsimony, heuristic search, and 500 bootstrap replicates (bootstrap values are shown on tree) and rooted with influenza virus type B isolate B/Lee/40. States are abbreviated by their standard two-letter postal codes.

acid, there are no substitutions in the M gene consistent with Amantadine resistance, and both the NA stalk region and NS1 proteins are full length.

Pathobiology in chickens and turkeys. A/Cinnamon Teal/Bolivia/4537/01 was determined to be a LPAIV based on the lack of clinical signs and mortality in specific-pathogen-free (SPF) chickens using the standard intravenous pathogenicity test (7). Infectivity of the virus for turkeys and chickens was determined by an intranasal mean (50%) bird infectious dose (BID_{50}) study using serial dilutions of virus and determining infection status based on seroconversion (8). A/Cinnamon Teal/Bolivia/4537/01 produced infections at a low challenge dose in turkeys (BID_{50}, $10^{1.1}$ EID_{50} [50% egg infectious doses]), indicating adaptation to turkeys, whereas chickens were more resistant to virus infection (BID_{50}, $10^{6.2}$ EID_{50}). Previously, chickens were shown to be experimentally resistant to infection with the H7N3 LPAIV recovered from chickens in the 2002 Chilean poultry outbreak (1).

TABLE 2. Antibody response and virus shed titers for 4-week-old SPF chickens and turkeys inoculated intranasally with A/Cinnamon Teal/Bolivia/4537/01

Species	Serology[a]	Virus isolation (2 dpi)[b]	
		Oropharyngeal swab[b]	Cloacal swab[b]
Chicken	2/8	2/8 (1.87)	1/8 (0.91)
Turkey	8/8	5/5 (4.22)	3/5 (1.46)

[a] Number positive/number tested by agar gel immunodiffusion test.
[b] Number positive/number tested. The mean titer in EID_{50}/ml of swab media of the positive birds is given in parentheses.

To further evaluate the pathobiology of A/CT/Bolivia/4537/01, ten 4-week-old SPF meat-type chickens and ten SPF turkeys were inoculated intranasally with 10^6 EID_{50} of virus. Eight birds were examined at 3 days postinoculation (dpi) for virus replication in the oropharynx and cloaca. No clinical signs or mortality were observed. Chickens were resistant to infection, as evidenced by only two birds developing AIV-specific antibodies by 14 dpi, and virus was shed from the oropharynx and cloaca in low titers (Table 2). In contrast, all turkeys became infected and most shed virus from the respiratory and intestinal tracts in higher titers (Table 2). Furthermore, moderate-to-severe lymphocytic rhinitis was observed, and AIV antigen was localized to the respiratory epithelium in the nasal cavity and infraorbital sinuses of two turkeys sampled on 3 dpi. Two chickens sampled at the same time had only mild lymphocytic rhinitis, and no AIV antigen was observed. In addition, passage of the virus through a 14-day-embryonating chicken-egg/adult-hen laboratory model system that has produced highly pathogenic AIV (HPAIV) from LPAIV in the past (8) failed to produce HPAIV with A/Cinnamon Teal/Bolivia/4537/01. Interestingly, during the 2002 AIV outbreak in Chilean poultry, the virus mutated from LPAIV to HPAIV virus within 1 month of its introduction into poultry (7), indicating that although this virus was LPAIV, it had the ability to become HPAIV when passaged in poultry.

An outbreak of H7N3 AIV in commercial poultry in Chile in 2002 is the only other reported isolation of AIV in South America (7). The source of the virus was postulated to be wild waterfowl because of low infectivity of the 2002 LPAIV for chickens (1), although there were no wild bird isolates available for genetic comparison at that time (this sample had been collected but not processed). The viruses from Chile were genetically divergent from other reported AIVs and were proposed to represent a separate South American AIV lineage (7), indicating that AIV has been circulating long enough in South America to evolve into a distinct lineage from North American viruses.

From an ecological standpoint there is no clear reason why AIV would not be present in South America as waterfowl, the natural reservoir for AIV (3), such as cinnamon teal and the closely related blue-winged teal (*Anas discors*), periodically migrate between North and South America, potentially providing a mechanism for intercontinental virus transfer. However, this particular cinnamon teal was identified as *Anas c. orinmous*, a subspecies of cinnamon teal that is nonmigratory and resident in the Central High Andes year-round (R. E. Wilson, unpublished data).

This virus from Bolivia, collected in 2001, about 9 months prior to the isolation of the H7N3 in commercial poultry in Chile, contains five genes (HA, NP, PA, PB1, and PB2) that are most closely related to the viruses from Chile, suggesting a common relative and supporting the theory that the virus in Chile was introduced by a wild bird. Importantly, the common ancestor of the Chilean virus and this virus may be years apart, which may explain the level of divergence between the isolates. Equally great divergence (10% or more) has been observed before among the polymerase wild-bird origin isolates within North America (unpublished data), and similar levels of divergence have been reported in wild bird isolates among the HA, M, NS, and NP genes of North American wild bird origin AIV isolates (5). However, the three remaining genes appear to be from diverse sources, indicating a reassortant virus. The NA and M were most closely related to North American wild aquatic bird virus lineages (5), suggesting that there is some exchange of AIV genes between North and South America. Finally, the NS gene was most closely related to NS genes from equine viruses. However, it is not clearly an equine lineage gene since the nucleotide divergence is greater than that among the other equine virus genes, and the NS genes of this lineages of equine viruses have been reported to be related to North American wild bird NS genes (2).

In general, the prevalence of AIV in wild aquatic birds likely varies among geographic regions because of the timing of migration, seasonal patterns of AIV in wild birds, and variation in AIV prevalence among different host species. Minimal historic sampling probably accounts for a lack of reported AIV in South American wild birds. As we continue surveillance of wild birds in South America for AIV, both the ecology and the genetics of AIV in wild birds will become better defined.

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REFERENCES

1. **Jones, Y. L., and D. E. Swayne.** 2004. Comparative pathobiology of low and high pathogenicity H7N3 Chilean avian influenza viruses in chickens. Avian Dis. **48:**119–128.
2. **Lindstrom, S., A. Endo, S. Sugita, M. Pecoraro, Y. Hiromoto, M. Kamada, T. Takahashi, and K. Nerome.** 1998. Phylogenetic analyses of the matrix and nonstructural genes of equine influenza viruses. Arch. Virol. **143:**1585–1598.
3. **Slemons, R. D., D. C. Johnson, J. S. Osborn, and F. Hayes.** 1974. Type-A influenza viruses isolated from wild free-flying ducks in California. Avian Dis. **18:**119–124.
4. **Spackman, E., D. A. Senne, T. J. Myers, L. L. Bulaga, L. P. Garber, M. L. Perdue, K. Lohman, L. T. Daum, and D. L. Suarez.** 2002. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J. Clin. Microbiol. **40:**3256–3260.
5. **Spackman, E., D. E. Stallknecht, R. D. Slemons, K. Winker, D. L. Suarez, M. Scott, and D. E. Swayne.** 2005. Phylogenetic analyses of type A influenza genes in natural reservoir species in North America reveals genetic variation. Virus Res. **114:**89–100.
6. **Suarez, D. L., M. Garcia, J. Latimer, D. Senne, and M. Perdue.** 1999. Phylogenetic analysis of H7 avian influenza viruses isolated from the live bird markets of the Northeast United States. J. Virol. **73:**3567–3573.

7. **Suarez, D. L., D. A. Senne, J. Banks, I. H. Brown, S. C. Essen, C. W. Lee, R. J. Manvell, C. Mathieu-Benson, V. Moreno, J. C. Pedersen, B. Panigrahy, H. Rojas, E. Spackman, and D. J. Alexander.** 2004. Recombination resulting in virulence shift in avian influenza outbreak, Chile. Emerg. Infect. Dis. **10:**693–699.
8. **Swayne, D. E., J. R. Beck, M. L. Perdue, M. Brugh, and R. D. Slemons.** 1996. Assessment of the ability of ratite-origin influenza viruses to infect and produce disease in rheas and chickens. Avian Dis. **40:**438–447.
9. **Swayne, D. E., D. A. Senne, and C. W. Beard.** 1998. Avian influenza, p. 150–155. *In* D. E. Swayne, J. R. Glisson, M. W. Jackwood, J. E. Pearson, and W. M. Reed (ed.), A laboratory manual for the isolation and identification of avian pathogens, 4th ed. American Association of Avian Pathologists, Kennett Square, Pa.