



## Sequence Analysis of the S3 Gene from a Turkey Reovirus

DARRELL R. KAPCZYNSKI,\* HOLLY S. SELLERS,† VALRIE SIMMONS  
& STACEY SCHULTZ-CHERRY

*Southeast Poultry Research Laboratory, Agricultural Research Service, United States  
Department of Agriculture, 934 College Station Road, Athens, Georgia, 30605.*

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**Abstract.** The deduced  $\sigma 2$  protein sequence from the S3 gene segment of a novel turkey reovirus, designated NC98, isolated from the bursa of birds exhibiting poult enteritis and mortality syndrome was determined. The isolate, serologically distinct from other avian reoviruses, was isolated in turkey embryo kidney cells and RNA was purified for cDNA synthesis. Oligonucleotide primers were designed based on conserved avian S3 nucleotide sequence data. The NC98 S3 open reading frame comprised 1101 base pairs and encoded 366 amino acids with a predicated molecular mass of 40.5 kDa. Although the S3 nucleotide sequence from several chicken isolates share at least 86% identity, they share only 64% with the NC98 turkey isolate. Interestingly, the S3 nucleotide sequence from a muscovy duck reovirus shares 55% identity with NC98 and 53% identity with chicken isolates. As observed in other avian reovirus  $\sigma 2$  protein sequences, a zinc-binding motif and double-stranded RNA binding domain were found within the predicted amino acid sequence of NC98. Phylogenetic analysis of the deduced  $\sigma 2$  sequence demonstrated that NC98 separated as a distinct virus relative to other avian strains. The results of this study indicate that NC98 is a novel turkey reovirus that shares limited genomic sequence identity to isolates of chicken and duck origin and should be considered a separate virus species within subgroup 2 of the *Orthoreovirus* genus.

**Key words:** avian pathogen, *Reoviridae*, S3 segment, turkey reovirus,  $\sigma 2$  protein

### Introduction

Avian reoviruses, along with mammalian reoviruses, comprise the genus *Orthoreovirus* in the family *Reoviridae*. These viruses contain 10 dsRNA genome segments enclosed within a non-enveloped, icosahedral double capsid of approximately 80 nm [15,29]. The genome segments can be separated based on electrophoretic mobility into three large (L1, 2, 3), three medium (M1, 2, 3) and four small (S1, 2, 3, 4) segments which code for proteins  $\lambda 1$ ,  $\lambda 2$ ,  $\lambda 3$ ,  $\mu 1$ ,  $\mu 2$ ,  $\mu NS$ ,  $\sigma 3$ ,  $\sigma 1$ ,  $\sigma 2$ ,  $\sigma NS$ , respectively [33,36,37]. Unlike

mammalian reoviruses, avian reoviruses do not hemagglutinate [8,24] but possess the ability to promote syncytial formation of cultured cells [22]. This biologic ability to induce cell–cell fusion can be used to phylogenetically classify orthoreoviruses into fusogenic and nonfusogenic groups [4]. Based on these studies, avian reoviruses separate from non-fusogenic mammalian reoviruses into a fusogenic clade along with the mammalian isolate, Nelson Bay virus (NBV). This clade also separates from a fusogenic baboon reovirus (BRV) isolate. The International Committee on Taxonomy of Viruses divides orthoreoviruses into the three distinct subgroups, nonfusogenic mammalian orthoreoviruses (subgroup 1), fusogenic avian and NBV orthoreoviruses (subgroup 2), and the baboon orthoreovirus (subgroup 3) [21]. Criteria for orthoreovirus species demarcation include nucleotide or amino acid identity of greater

\*Author for all correspondence:

E-mail: dkapczynski@seprl.usda.gov

†Present address: Department of Avian Medicine, Poultry Diagnostic and Research Center, College of Veterinary Medicine, University of Georgia, 953 College Station Road, Athens, Georgia, 30602.

than 75% and 85%, respectively, within a species, or less than 60% and 65%, respectively, between a species [21].

Differences in genomic coding sequences for individual proteins exist between avian and mammalian reoviruses. For example, the S1 segment encodes the outer capsid protein  $\sigma 3$  of avian reoviruses which is structurally analogous to the mammalian reovirus  $\sigma 1$  protein and appears to be responsible for cell-attachment [18,29,31,34,38]. Likewise, the S3 segment of avian reovirus encodes the  $\sigma 2$  protein and is analogous to the mammalian reovirus S4 segment encoding the  $\sigma 3$  protein. Other investigators have identified distinct binding motifs for zinc at the amino-terminal and dsRNA at the carboxy-terminal contained in the avian  $\sigma 2$  protein and mammalian  $\sigma 3$  protein [17,28,35,39].

Poult enteritis and mortality syndrome (PEMS) is a highly infectious disease of turkey poults. The disease is characterized by diarrhea, decreased growth, increased morbidity and mortality, immunosuppression and metabolic dysfunction [1,5,6,12,23,30]. Since its emergence during 1991, the disease has caused severe economic losses to the turkey industry. The cause of PEMS is unknown, however, as new etiologic agents are isolated from birds with PEMS it appears that the disease may be multifactorial. Efforts to identify the causative agent of PEMS has resulted in identification of several potential infectious agents including viruses, bacteria and protozoa [1,3,5–7,9,10,19]. Avian reoviruses have been isolated from turkeys with PEMS, as well as chickens and ducks, and are associated with enteric and respiratory disease, viral arthritis/tenosynovitis, malabsorption and stunting syndrome [25]. Despite being an important poultry pathogen very little molecular data is available on avian reoviruses and in particular turkey reoviruses. The aim of this study was to present new sequence information for the avian reovirus S3 segment and examine sequence diversity among avian isolates. This represents the first report of genomic sequence from a reovirus of turkey origin and provides further insight into the  $\sigma 3$  protein sequence.

The NC98 strain of turkey reovirus was isolated from the bursa of turkeys experiencing PEMS in North Carolina during 1998. Briefly, bursas were homogenized in PBS and passed through a 0.2  $\mu$ M GDX filter (Whatman, Fisher Scientific, Norcross, GA). The bursal filtrate was used to inoculate specific-pathogen-free (SPF) turkey embryo kidney

cells. Following the second passage, syncytial cell formation consistent with reovirus was observed among infected cells. Isolation was confirmed by electron microscopy and indirect immunofluorescence using antisera against the chicken reovirus S1133 (data not shown). Following plaque purification and propagation, RNA was purified using Trizol<sup>®</sup> Reagent (BRL-Life Technologies, Gaithersburg, MD) according to the manufacturers recommendations. Primers to the S3 segment (ReoS3F, 5' ATGGAGGTACGTGTGCCAAACT and ReoS3R, 5' CCAACCACACTCCACAACAG) were designed using Oligo<sup>®</sup> 4.0 (National Biosciences, Inc., Plymouth, MN) based on conserved nucleotide sequences from previously reported S1133 avian reovirus S3 sequence. A cDNA corresponding to the S3 gene was produced by reverse transcription and PCR using Superscript<sup>™</sup> II RNase H-reverse transcriptase and Platinum<sup>®</sup> Taq DNA polymerase (Invitrogen, Carlsbad, CA) with both ReoS3 primers. The amplified products were separated on a 0.8% agarose gel and a 1.1 kb size fragment was excised and purified with QIAEX II gel extraction kit (Qiagen Inc., Valencia, CA). The S3 gene was cloned into plasmid pCR2.1 (Invitrogen) and transformed into *Escherichia coli* according to the manufacturers recommendations. Several clones containing plasmids with the 1.1 kb insert were identified by restriction enzyme analysis. The clones were amplified and the plasmid DNA purified using Qiagen plasmid miniprep kit (Valencia, CA) according to methods of the manufacturer.

Double-stranded DNA sequencing [27] with fluorescently labeled dideoxynucleotides and Taq polymerase (Applied Biosystems Inc., Foster City, CA) was performed with an automated sequencer [32] and M13 universal forward and reverse primers. Internal NC98 S3 gene primers were produced as needed to complete sequencing (available upon request). Nucleotide, predicted amino acid sequence analysis and multiple alignments were performed with the CLUSTAL V [13] method using LASERGENE software version 1.03 (DNASTAR, Madison, WI). An unrooted phylogenetic tree was generated using the maximum parsimony method with a neighbor-joining search [26] utilizing the Phylogenetic Analysis Using Parsimony (PAUP 4.0b) software program and 2000 bootstrap replications [11].

At least three clones from three amplifications containing the putative NC98  $\sigma 2$  nucleotide sequences were analyzed. The nucleotide and predicted amino

acid sequence for the NC98  $\sigma 2$  protein represents a consensus sequence from three different clones. The S3 open reading frame encodes a protein of 366 amino acids with a predicted molecular weight of 40,257 Da and an isoelectric point of 6.21 with a  $-6.09$  charge at pH 7. The number of nucleotides contained in the S3 open reading frame differed between NC98 and other reported isolates. While the NC98 turkey isolate was determined to contain 1101 nucleotides, the chicken and duck reovirus isolates contained 1104. This results in a decrease of 1 amino acid in the NC98 isolate, presumably at amino acid position 307. This

could account for a slight decrease in molecular weight between NC98 and other avian  $\sigma 2$  proteins (Fig. 1). The TAA stop codon of NC98 was shared with isolates S1133, 1733, 138 and 176 of chicken origin, however, the duck muscovy isolate 89026 utilizes a TAG stop codon [17].

The alignment of NC98 S3 nucleotide sequence was compared with other subgroup 2 orthoreoviruses. The NC98 isolate shared only 64% nucleotide sequence identity with chicken isolates S1133, 1733, 138, 176 and 55% identity to duck 89026 (data not shown). Chicken viruses shared 87–99% identity

NC 98	M E V R V P N F H S F V E G I T S S Y I R A P A C W N A K T M W D V E T F H L P D V I K V G N A Y C	50
1133	. . . . . L K T . . . . . Q . A . . T V . . . V . . . R . . . . .	50
1733	. . . . . L K T . . . . . Q . A . . T V . . . V . . . R . . . . .	50
138	. . . . . L Q T . . . . . Q . A . . T V . . . V . . . R . . . . .	50
176	. . . . . L K T . . . . . Q . A . . T V . . . V . . . R . . . . .	50
duck	. . . . . A . . . . . T . . . . . D S R S A . . S D I . Y E . . . . . G . . . . .	50
NC 98	<u>C S Q C C G V L Y Y G A P P S D G N Y F P H H K C H</u> Q Q Q Y R S D T P L L R Y V R I G S T T E H L L	100
1133	. . . . . T L . A . . . . . T . . . . . T . . . . . R . . . . .	100
1733	. . . . . T L . A . . . . . T . . . . . T . . . . . R . . . . .	100
138	. . . . . T L . A . . . . . F . T . . . . . R . . . . .	100
176	. . . . . T L . A . . . . . T . . . . . T . . . . . R . . . . .	100
duck	. T . . . . . T . . . . . I C . . . . . L S . V . N . . . . . F . K . . . . . R . . . . .	100
NC 98	D Q Y A V A L Q T I A D Y Y D E A S H R V A D E A E E D T I A A L D I V T R T E S I R G D Q A V D A	150
1133	. . . . . E S . . H . . I . Q . M V . . P . N . E V . P . . . . . S . K T . . . P	150
1733	. . . . . E S . . H . . I . Q . M V . . P . N . E V . P . . . . . S . K T . . . P	150
138	. . . . . E S . . E H . . I . Q . M V . . P . N . E V T P . . . . . S . K T . . . P	150
176	. . . . . E S . . H . . I . Q . M V . . P . N . E V . P . . . . . S . K T . . . P	150
duck	. . . . . I . S C . . . H . E Q . A R S R T S M C G G . A L M S . . . . . I M . . . . . L . T . R P . . . P	150
NC 98	D F W T Y P L E R R S D D S R R D I A A S I W T M I D A S A R S F T L P E C L V S P S L H S R H I F	200
1133	. . . . . R . . . . . S . . . . . L . . . . . N . . . . . S . V . . . . .	200
1733	. . . . . R . . . . . C . R . . . . . S . . . . . L . . . . . N . . . . . S . V . . . . .	200
138	. . . . . Q . . . . . N . . . . . S . C . R . . . . . S . . . . . L . . . . . N . . . . . V . . . . . S . V . . . . .	200
176	. . . . . R . . . . . C . R . . . . . S . . . . . L . . . . . N . . . . . S . V . . . . .	200
duck	. . . . . A . . . . . D . . . . . E P . A . S . . . . . T A G . R . . . . . S Q . Q . V . D . V . . . . . N L . . . . . T . . . . . V . . . . .	200
NC 98	D Q M L T T T S I Y D V A A S G K T A R F S P M V A A L P Q R T A G P I T L P D A D P F D G V A T F	250
1133	G . . Q . . . T . . . . . A V K . . . . . T . S . . . . . D . . . . . V M . A N . . . . . A E . . . . . Y S . . . . .	250
1733	G . . Q . . . T . . . . . A V K . . . . . T . S . . . . . D . . . . . V K . A N . . . . . A E . . . . . Y S . . . . .	250
138	G . . Q . . . T . . . . . T . . . . . A V K . . . . . T . A . . . . . D . . . . . V M . A N . . . . . A . . . . . Y S . . . . .	250
176	G . . Q . . . T . . . . . A V K . . . . . T . S . . . . . D . . . . . V K . A N . . . . . A E . . . . . Y S . . . . .	250
duck	S . . . . . T A . . . . . V T . . . . . P . K . . . . . L . . . . . V M . T . D S . I . S . T R D N W D H D . E G V	250
NC 98	W S P Q F A L S P M I G G V G I T G Q Y A R E S Y H H V G H P V I G S G <u>K K V S H Y R</u> N L F M D A W	300
1133	. T S H . . F . . L . . . . . L . . . . . A . . . . . A . . . . . E S . . . . .	300
1733	. T S H . . F . . L . . . . . L . . . . . A . . . . . A . . . . . E S . . . . .	300
138	. T S H . . F . . L . . . . . L . . . . . A . . . . . A . . . . . E . . . . .	300
176	. T S H . . F . . L . . . . . L . . . . . A . . . . . A . . . . . E S . . . . .	300
duck	. L N G . . F . . I . . . . . S . . . . . F E . G A C . N Y . . . . . M V . . . . . A . . . . . E V C . . . . .	300
NC 98	R G W S K S - F T C A A G L E P A E C E S R L R G H A R T M L G R S L P G V C D C G P E A Q S R T A	349
1133	. . . . . A . A . . T . M . . . . . N . . . . . D E V A Q . . G A V	350
1733	. . . . . A . A . . T . M . . . . . N . . . . . D E V A Q . . G A V	350
138	. . . . . A . A . . T . M . . . . . N . . . . . D D V A Q . . G A V	350
176	. . . . . A . A . . T . M . . . . . N . . . . . D E V A Q . . G A V	350
duck	. . . . . R . S . . . . . V . . . . . L . . . . . Q . . . . . A . . . . . D . . . . . F D E T T H T G Q S	350
NC 98	P S S L Q K A T K L T V V E C G W	366
1133	L T . . . . . T . . . . . F . E . . . . .	367
1733	L T . . . . . T . . . . . F . . . . .	367
138	L A . . . . . T . . . . . F . . . . . S . . . . .	367
176	L T . . . . . T . . . . . F . . . . .	367
duck	S A P . R R S S . V S F I . . . . .	367

Fig. 1. Alignment of predicated open reading frame amino acid sequences of avian reovirus S3 gene  $\sigma 2$  proteins. Sequences from turkey (NC98-GenBank accession no. AF465799), chicken (S1133-AO020642, 1733-AF004856, 138-AF059721, 176-AF059720), and duck (89026-AJ006476) reoviruses. The putative zinc-finger binding-motif is underlined (amino acids 51–76) and the proposed dsRNA-binding motif is boxed (amino acids 291–297).

among isolates and approximately 53% identity with the duck strain. Alignment of the predicated amino acid sequence for  $\sigma_2$  protein resulted in only 77% identity of the turkey isolate with isolates S1133, 1733, 138, 176 and only 60% amino acid identity with duck 89026, respectively (Fig. 1). The  $\sigma_2$  protein amino acid sequences of the chicken isolates examined share between 95% and 100% identity. These strains share approximately 61% identity with duck 89026. Comparisons between NC98 and NBV revealed only 33% nucleotide and 31% amino acid sequence identity. The NC98 isolate shared approximately 25% and 14% nucleotide and amino acid identity, respectively, with the BRV and human reovirus Lang strain (serotype 1)  $\sigma_3$ , which is similar to what was previously reported for chicken [35] and duck reoviruses [17].

Within the NC98  $\sigma_2$  amino acid sequence a conserved CHCC zinc-binding motif was identified within the N-terminus, at amino acids 51–76 [17,28]. These motifs are thought to be involved in binding to nucleic acids [2] and increase the stability of reoviruses [20]. Nine cysteine and histidine residues were identified within amino acid positions 38–76, which may serve as ligands for zinc ions [28]. Le Gall-Recule et al. [17] reported a C-X<sub>2</sub>-C-X<sub>17</sub>-H-X<sub>2</sub>-C motif within the duck reovirus  $\sigma_2$  amino acid sequence, which is similar to the proposed mammalian  $\sigma_3$  motif C-X<sub>2</sub>-C-X<sub>15</sub>-H-X<sub>2</sub>-C. Our results indicate that the NC98 isolate shares the motif found in both duck and chicken reovirus strains. Also, a putative double stranded (ds)-RNA binding motif, reported to contain numerous basic amino acids, was identified within the  $\sigma_2$  C-terminus at amino acid positions 291–297 [16,28]. Research performed with mammalian reoviruses indicates that the basic amino acid motifs may sequester dsRNA away from cellular protein kinase, possibly inhibiting interferon production by the host cell [14,16,20]. Although the functional properties of the avian reovirus  $\sigma_2$  protein remains to be determined, based on the predicated amino acid motif similarities they would appear to have similar functions.

Phylogenetic analysis of the NC98  $\sigma_2$  protein amino acid sequences demonstrated that the turkey reovirus isolate separates from subgroups 1 and 3 orthoreovirus isolates, and separates within subgroup 2 orthoreoviruses (Fig. 2). Within the subgroup 2 orthoreoviruses, NC98 separates between the duck and chicken isolates. This relationship was also

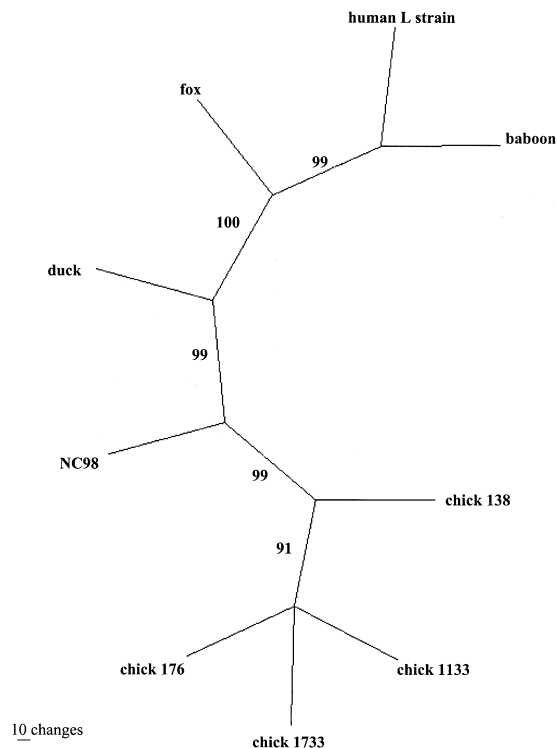


Fig. 2. Phylogenetic relationships among avian reoviruses based on  $\sigma_2$  amino acid sequences. An unrooted phylogenetic tree was constructed following amino acid alignment by neighbor-joining method using parsimony analysis with the mammalian reovirus isolates, human L strain (GenBank accession no. M13139), baboon (AF406787) and fox (Nelson Bay-AF059722) S4 coding sequence. Bootstrap confidence levels following 2000 replicates are listed next to each branch.

determined using nucleotide coding sequences of the  $\sigma_2$  gene among subgroup 2 orthoreoviruses and was supported by high bootstrap confidence levels (data not shown). Based on the phylogenetic analysis and sequence identity at the nucleotide and protein level between NC98 and the avian and NBV isolates, it is proposed that the turkey isolate is a different species from the chicken and NBV isolates within the subgroup 2 clade.

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