# Investigating Turkey Enteric Coronavirus Circulating in the Southeastern United States and Arkansas During 2012 and 2013

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*Research Note—*

# Investigating Turkey Enteric Coronavirus Circulating in the Southeastern United States and Arkansas During 2012 and 2013

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SUMMARY. Periodic monitoring of poultry flocks in the United States via molecular diagnostic methods has revealed a number of potential enteric viral pathogens in continuous circulation in turkeys and chickens. Recently turkey integrators in the Southeastern United States and Arkansas experienced an outbreak of moderate to severe enteritis associated with turkey enteric coronavirus (TCoV), and numerous enteric samples collected from turkey flocks in these areas tested positive for TCoV via real-time reverse-transcriptase PCR (RRT-PCR). This report details the subsequent sequence and phylogenetic analysis of the TCoV spike glycoprotein and the comparison of outbreak-associated isolates to sequences in the public database. TCoVs investigated during the present outbreak grouped geographically based upon state of origin, and the RRT-PCR assay was a good indicator of subsequent seroconversion by TCoV-positive turkey flocks.

RESUMEN. *Nota de Investigación*—Investigación de coronavirus entéricos de pavos que estaban circulando en el sureste de los Estados Unidos y en Arkansas durante los años 2012 y 2013.

El muestreo periódico de parvadas avícolas en los Estados Unidos mediante métodos de diagnóstico molecular ha revelado una serie de patógenos vírales entéricos potenciales que circulan continuamente en pavos y pollos. Recientemente los integradores de pavo en el sureste de los Estados Unidos y en Arkansas experimentaron un brote de enteritis de moderada a severa asociada con un coronavirus entérico de los pavos (TCoV), numerosas muestras entéricas recolectadas de las parvadas de pavos en estas áreas resultaron positivas al coronavirus de los pavos mediante transcripción reversa y PCR en tiempo real (RRT -PCR). Este reporte detalla la secuenciación y el análisis filogenético de la glicoproteína de las espículas del coronavirus de los pavos y compara los aislamientos asociados con brotes con las secuencias de una base de datos pública. Los coronavirus de pavos analizados durante un brote reciente se agruparon geográficamente en función del estado de origen. El ensayo de PCR en tiempo real fue un buen indicador de la seroconversión posterior de los lotes positivos al coronavirus de los pavos.

Key words: turkey, enteric, coronavirus, phylogenetics

Abbreviations: IBV = infectious bronchitis virus; PEC = poult enteritis complex; PEMS = poult enteritis mortality syndrome; RRT-PCR = real-time reverse-transcriptase PCR; SEPRL = Southeast Poultry Research Laboratory; TCoV = turkey enteric coronavirus

Turkey enteric coronavirus (TCoV) causes a contagious form of enteritis in turkeys, generally recognized in the field by outward signs including diarrhea and decreased weight gain (10). TCoV has historically caused severe economic losses for the poultry industry and in the past was associated with poult enteritis mortality syndrome (PEMS), which caused an enteritis with markedly increased mortality and growth depression as well as immune dysfunction (1). The seasonality and cyclical nature of recognized enteric disease syndromes, such as poult enteritis complex (PEC) in young turkeys and runting-stunting syndrome in broiler chickens, warrants ongoing surveillance for enteric viruses that may not currently be suspected in performance problems in the field. Beginning in the summer of 2012 in the Southeastern United States and Arkansas, a moderate to severe enteritis appeared in numerous turkey flocks; this enteritis had the hallmarks of a TCoV infection and was highly transmissible. By October 2012, numerous turkey farms in Arkansas reported severe flushing with suspected TCoV involvement; by the end of October 2102 alone, more than 60 individual farms had reported cases of this suspected TCoV enteritis. The first positive reverse-transcriptase PCR (RT-PCR) results for TCoV were noted in mid-October, with serology via ELISA confirming these results by the end of October 2012. In Arkansas, the majority of TCoV-positive cases were in turkeys between 5 and 9 weeks of age, but the outbreak included birds as young as 3 weeks and as old as 14 weeks of age. A similar outbreak began in North Carolina in the Spring of 2012, with numerous isolated farms reporting suspected cases by the end of July 2012. Common themes between these two concurrent outbreaks were the involvement of multiple companies, and the often-isolated appearance of a TCoV-positive flock many miles from previous positive flocks, with no clear mode of dispersal. Subsequently, a number of turkey enteric field samples received at the Southeast Poultry Research Laboratory (SEPRL) in Athens, GA were found to be positive for TCoV via a real-time RT-PCR (RRT-PCR) assay developed at SEPRL (20). Subsequent nucleic acid sequencing of a portion of the coronavirus spike glycoprotein gene revealed that the turkey coronaviruses

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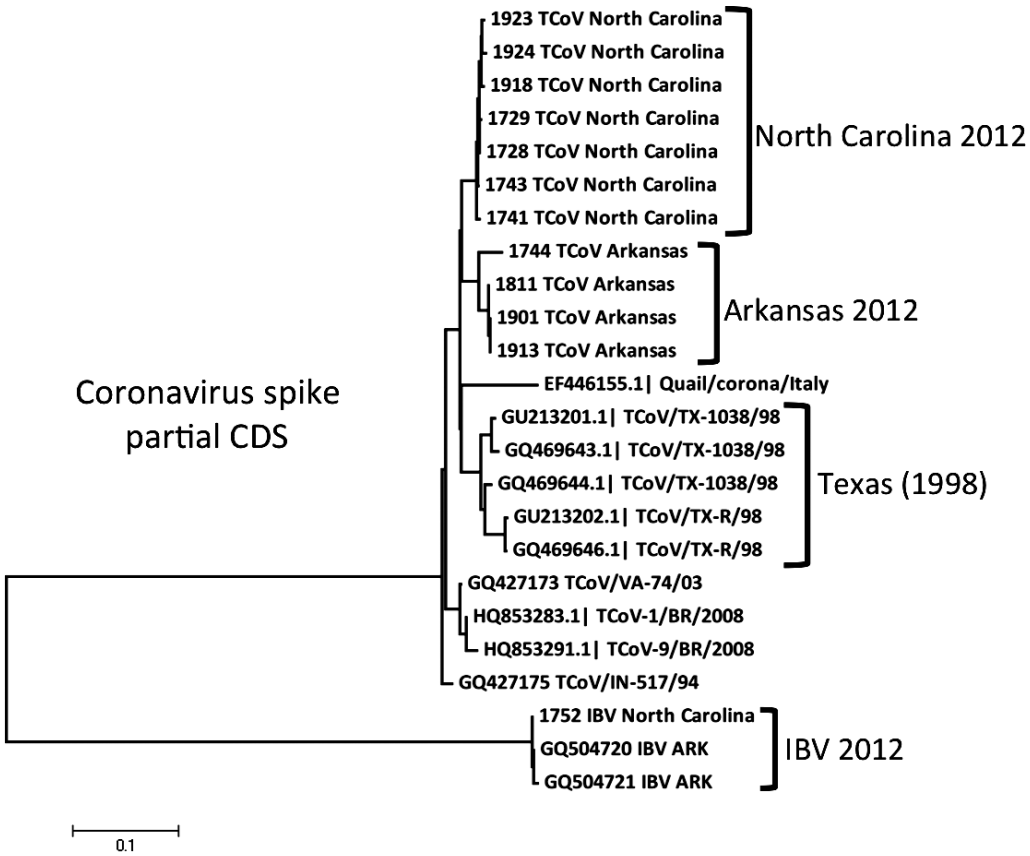


Fig. 1. Neighbor-joining tree comparing the TCoV spike glycoprotein partial coding sequence prepared using MEGA4. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the tree.

currently circulating in the Southeastern United States and Arkansas are unique when compared to available turkey coronavirus sequences in public databases and when compared to each other. Further—based upon an initial sequence analysis—infectious bronchitis virus (IBV) coronaviruses isolated in the Southeastern United States during the same time period, and in an adjacent geographical area to the active TCoV outbreak, did not appear to be associated with the enteric coronaviruses circulating in turkeys during this outbreak.

## MATERIALS AND METHODS

**Receipt and handling of enteric samples.** Frozen turkey intestinal samples (either whole intestines or a section comprising the duodenum/pancreas) were shipped on wet ice to SEPRL in Athens, GA via overnight courier and were promptly homogenized via blending into 10% homogenates in phosphate-buffered saline (PBS) and clarified via centrifugation (2400 × *g*) for 15 min at 4 C. Total RNA was extracted from 250 µl of clarified supernatant using a hybrid RNA extraction protocol with TRIzol® LS reagent (Life Technologies, Grand Island, NY) followed by use of a commercially available extraction kit (Ambion Mag Max Viral RNA extraction kit, Life Technologies), as previously described (5,19). The first samples ($n = 2$) associated with the recognized outbreak in the field were received in July 2012 at SEPRL; the gut samples were collected from North Carolina turkey farms in June 2012. Also in July 2012, embryonated turkey egg-passaged samples originally collected from suspect TCoV-positive North Carolina turkey flocks were received at SEPRL (T. Hooper, Purdue University). The North Carolina turkey enteric sample, which was analyzed using a metagenomic approach, was handled and prepared as previously described to generate cDNA representing the gut virome (6); it was then sequenced using the Ion Torrent Personal Genome Machine (PGM) platform and associated chemistries (Life Technologies) and analyzed using the Metagenomics RAST (MG-RAST) server (18). Suspected North Carolina IBV isolates collected from broiler farms in the summer of 2012 were received from the Georgia Poultry Laboratory, Oakwood, GA (B. Johnson, A. Kulkarni) for comparison to TCoV sequences.

**Real-time and conventional RT-PCR for TCoV and sequence analysis.** The diagnostic screening of enteric field samples for the presence of TCoV was performed using a previously described RRT-PCR assay targeting a 112-bp portion of the TCoV matrix (M) gene (20). The TCV 2F/TCV 112R primers and the TCV 51PB probe were included in a singleplex reaction and run on a BioRad CFX96 Real Time system (Bio-Rad, Hercules, CA). Selected purified RNA was chosen for subsequent conventional RT-PCR with primers targeting the coronavirus spike glycoprotein gene. The primers TCoV S1 5′ and TCoV S1 3′ were used to amplify an approximately 1.7-kb portion of the spike glycoprotein gene (14). RT-PCR amplicons were cloned into the pCR2.1 TOPO vector (Invitrogen, Grand Island, NY) and were subsequently sequenced in both directions at the USDA/ARS South Atlantic Area Sequencing Facility using the M13 forward and reverse primers (Applied Biosystems BigDye® Terminator v1.1 cycle sequencing kit and 3730 DNA Analyzer, Life Technologies). RT-PCR primers targeting the IBV genome (M41 spike 1, 5′-AAC TGA ACA AAA GAC AGA C-3′; M41 spike 1875, 5′-TAT CCA TAC GCG TTT GTA TG-3′) were designed to amplify a portion of the IBV spike glycoprotein gene. The North Carolina IBV isolates were successfully amplified using the M41 spike 1/M41 spike 1875 primers. Real-time and conventional RT-PCR reactions utilized the Qiagen One-Step RT-PCR kit (Qiagen, Inc., Valencia, CA). After RT-PCR and sequencing, the TCoV and IBV sequences were assembled using SeqMan (DNAStar®, Inc., Madison, WI) and multiple alignments were generated with ClustalW (12) and phylogenetic comparisons were completed using MEGA4 (21).

**Serology of suspected TCoV-positive flocks.** A competitive ELISA utilizing the TCoV nucleocapsid (N) protein for the detection of

Table 1. Sample information for TCoV sequences included in the phylogenetic tree in Figure 1.

| SEPRL accession no. | Source | State of origin | Collection date | Notes |
|---|---|---|---|---|
| 1728 | Integrator 1 | North Carolina | June 2012 | |
| 1729 | Integrator 1 | North Carolina | June 2012 | |
| 1741 | Integrator 1 | North Carolina | June 2012 | Egg-passed material |
| 1743 | Integrator 1 | North Carolina | June 2012 | Egg-passed material |
| 1744 | Integrator 1 | Arkansas | July 2012 | |
| 1811 | Integrator 2 | Arkansas | October 2012 | |
| 1901 | Integrator 1 | Arkansas | November 2012 | |
| 1913 | Integrator 1 | Arkansas | November 2012 | |
| 1918 | Integrator 3 | North Carolina | December 2012 | |
| 1923 | Integrator 3 | North Carolina | December 2012 | |
| 1924 | Integrator 1 | North Carolina | January 2013 | |

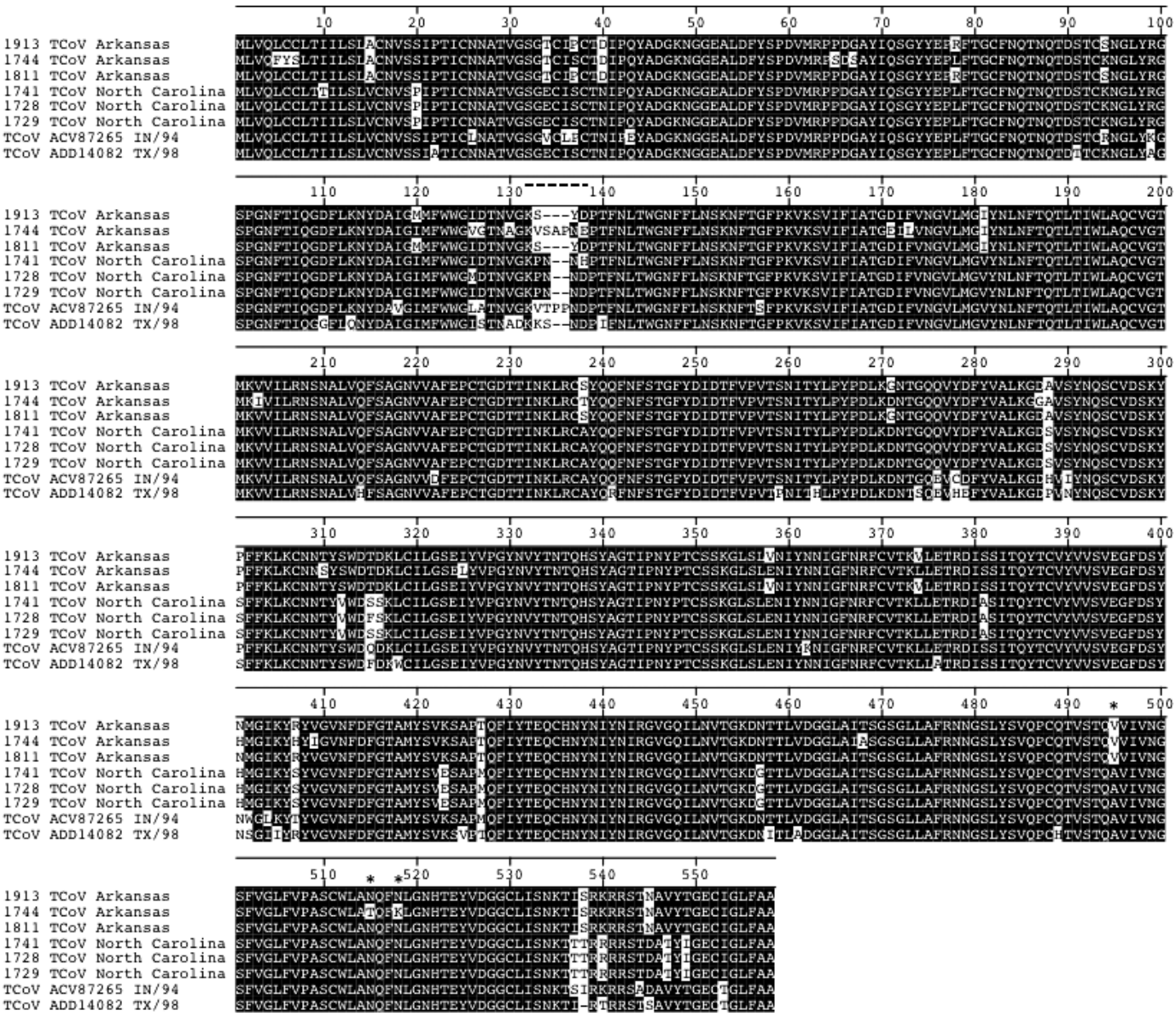


Fig. 2. Amino acid alignment of the S1 portion of the TCoV spike glycoprotein. Selected isolates from North Carolina and Arkansas were aligned with similar TCoV isolates from Texas and Indiana (nucleotide accession numbers GU213201.1 and GQ427175, respectively, in the phylogenetic tree in Fig. 1). Variable region referred to in the text is indicated with a dashed line; variable amino acids within a putative neutralizing-epitope–containing region are indicated with asterisks. North Carolina and Arkansas isolates are deposited in GenBank with accession numbers KJ146015–KJ146020.

Table 2. Samples received from integrator 2 (Arkansas origin) that were positive for TCoV RNA via the RRT-PCR assay and the results of up to three TCoV-specific ELISAs performed on the corresponding flocks. Dates assays were performed are indicated.[A]

| SEPRL accession no. | RT-PCR | ELISA 1 | ELISA 2 | ELISA 3 |
|---|---|---|---|---|
| 1800 | + (10/24/12) | suspect (10/26/12) | + (11/26/12) | N/A |
| 1801 | + (10/24/12) | + (11/14/12) | N/A | N/A |
| 1802 | + (10/24/12) | N/A | N/A | N/A |
| 1803 | + (10/24/12) | + (10/24/12) | + (11/26/12) | N/A |
| 1810 | + (10/30/12) | − (11/05/12) | − (11/14/12) | − (11/26/12) |
| 1811 | + (10/30/12) | − (11/05/12) | − (11/14/12) | + (11/26/12) |
| 1812 | + (10/30/12) | + (11/06/12) | + (11/14/12) | N/A |
| 1813 | + (10/30/12) | + (11/06/12) | N/A | N/A |
| 1814 | + (10/30/12) | + (11/06/12) | N/A | N/A |
| 1817 | + (10/30/12) | + (11/06/12) | N/A | N/A |
| 1818 | + (10/30/12) | − (11/14/12) | N/A | N/A |
| 1819 | + (10/30/12) | − (11/05/12) | − (11/14/12) | N/A |
| 1820 | + (10/30/12) | − (11/05/12) | + (11/14/12) | N/A |
| 1823 | + (10/30/12) | − (11/14/12) | − (11/26/12) | N/A |
| 1834 | + (10/30/12) | − (11/05/12) | + (11/26/12) | N/A |
| 1836 | + (10/31/12) | + (11/06/12) | N/A | N/A |
| 1837 | + (10/30/12) | + (11/06/12) | N/A | N/A |
| 1842 | + (10/30/12) | − (11/14/12) | − (11/26/12) | N/A |
| 1843 | + (10/30/12) | + (11/26/12) | N/A | N/A |
| 1859 | + (11/09/12) | − (11/26/12) | N/A | N/A |
| 1868 | + (11/15/12) | + (11/14/12) | N/A | N/A |
| 1873 | + (11/15/12) | − (11/14/12) | + (11/26/12) | N/A |
| 1874 | + (11/15/12) | − (11/14/12) | − (11/26/12) | N/A |

[A](+) = positive; (−) = negative; N/A = not applicable.

TCoV antibodies in suspect flocks was performed as previously described (11).

## RESULTS AND DISCUSSION

The North Carolina TCoV isolates 1728, 1729, 1741, 1743, 1918, 1923, and 1924 (SEPRL accession numbers) grouped together in a neighbor-joining tree but were distinct from other TCoV sequences from Texas, Virginia, Indiana, and Brazil for which there are spike glycoprotein sequences in the database (Fig. 1). These field samples were received from two different turkey integrators in North Carolina (Table 1), and the accession numbers 1741 and 1743 represent embryonated turkey egg-passaged material (embryo gut homogenates). Arkansas TCoVs 1744, 1811, 1901, and 1913 also grouped together and were distinct from the North Carolina isolates collected during the same period of time (2012). It is important to note that isolates from different companies in each state still grouped very closely to one another. The Arkansas field samples were also received from two separate turkey integrators (Table 1). Further, the North Carolina IBV isolate (1752) did not group closely with any of the TCoV sequences but did group closely with IBV Arkansas DPI vaccine strains (Fig. 1). Concerns were raised by field veterinarians during the North Carolina TCoV outbreak that, perhaps, the circulating TCoV in turkeys was related in some way to variant IBV in broilers that was observed at the same time and in adjacent broiler-producing areas in North Carolina. This concern was based upon previously obtained molecular evidence that suggested the emergence of TCoV within the group 3 coronaviruses was facilitated by a recombination event that replaced the spike glycoprotein gene of IBV with the spike glycoprotein of an unidentified coronavirus, leading to the enteric tropism and cross-species transmission of TCoV (9,14,15,16). The present phylogenetic analysis does not support a cross-species transmission event as the source of the Southeastern and Arkansas TCoV outbreak, and the reservoir for these TCoVs remains unknown.

The nucleic acid sequences from selected isolates were translated and the portion of the spike glycoprotein corresponding to the S1 subunit was aligned using ClustalW (12). There was 94.0% to 99.6% amino acid identity observed among the Arkansas isolates while amino acid identities ranged from 98.0% to 99.6% for the North Carolina isolates. Isolate 1729 (NC) and isolate 1913 (AR) shared the highest amino acid identity between states (94.0%) while isolates 1744 (AR) and 1728 (NC) shared the lowest (91.0%). The amino acid identity noted between geographic isolates (NC and AR) was similar to the range of identities observed between any of the isolates included in the present study and similar isolates in the database from Indiana and Texas (ranging from 89.7% to 93.2%). Further, representative Arkansas and North Carolina isolates showed a marked variability in a region spanning amino acid positions (approximately) 130 to 140 (Fig. 2) of the spike gene, which lies within a previously reported hypervariable region in TCoV (17). The Arkansas isolates had amino acid differences within a neutralizing-epitope–containing fragment (4) within the spike protein: isolates 1913 and 1811 had a valine (Val) at amino acid position 492 and isolate 1744 had a Val at the corresponding position 495, while the North Carolina isolates had an alanine. Further, isolate 1744 had a threonine and a lysine at positions 515 and 518, respectively, while all other representative isolates had a asparagine at each position.

The flocks from which the enteric field samples were collected from a single turkey integrator (integrator 2 in Table 1) in Arkansas during the fall of 2012 were also each assayed via a TCoV-specific ELISA (11)—sometimes as many as three times during the ongoing outbreak. Of the Arkansas turkey flocks that were positive for TCoV RNA via the RRT-PCR assay ($n = 23$), 74% of the flocks eventually seroconverted (Table 2). An additional 22 samples collected from the same Arkansas integrator were negative for TCoV RNA via the RRT-PCR assay; of these negative samples, one eventually seroconverted (4.5%). In some cases, if obvious TCoV enteritis was present in a flock and a positive RRT-PCR result was obtained,

a subsequent blood sample for ELISA was not collected and the flock was treated as TCoV positive from a management and biosecurity standpoint. An enteric sample positive for TCoV RNA via RRT-PCR appeared to be a good predictor of a subsequent seroconversion of a flock in the field and is a very sensitive and specific assay to assess TCoV infection and shedding (3,20).

Finally, deep sequencing and metagenomic analysis of the North Carolina isolate 1728 (see Fig. 1) confirmed the presence of TCoV RNA and indicated the presence of viral RNA from the members of the *Picornaviridae* family as well. The presence of enteric picornavirus in the poultry gut is not surprising given the recent characterization of novel and unclassified picornaviruses from the avian gut (2,6,7,8,22), but the role the enteric picornaviruses might play in enteric disease—or if the presence of picornavirus might exacerbate a TCoV infection—is not known. The effect of a TCoV infection on the absorptive function of the turkey gut was exacerbated by the presence of turkey astrovirus (13). Experimental work remains to be done to explore the combined effects of concomitant enteric viral infections on poultry performance and disease status.

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