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Investigating Turkey Enteric Picornavirus and Its Association with Enteric Disease in Poults

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SUMMARY. Previous research into the viral community in the poultry gastrointestinal tract has revealed a number of novel and partially described enteric viruses. It is evident that the poultry gut viral community remains minimally characterized and incompletely understood. Investigations into the microbiome of the poultry gut have provided some insight into the geographical distribution and the rapidly evolving taxonomy of the avian enteric picornaviruses. The present investigation was undertaken to produce a comparative metagenomic analysis of the gut virome from a healthy turkey flock versus a flock placed in the field. This investigation revealed a number of enteric picornavirus sequences that were present in the commercial birds in the field that were completely absent in the healthy flock. A novel molecular diagnostic assay was used to track the shedding of field strains of turkey enteric picornavirus in commercial poults inoculated with picornavirus-positive intestinal homogenates prepared from turkeys that were experiencing moderate enteric disease. The propagation of this novel enteric picornavirus in commercial poults resulted in significant reduction in weight gain, and suggests that this common inhabitant of the turkey gut may result in performance problems or enteric disease in the field.

RESUMEN. Investigaciones sobre picornavirus entéricos de los pavos y su asociación con enfermedad entérica en pavipollos.

Las investigaciones anteriores acerca de la comunidad viral en el tracto gastrointestinal de las aves comerciales han revelado una serie de virus entéricos nuevos y parcialmente descritos. Es evidente que la comunidad viral en el intestino de las aves comerciales permanece mínimamente caracterizada y no se comprende completamente. Las investigaciones sobre el microbioma del intestino de las aves comerciales han proporcionado alguna información sobre la distribución geográfica y la taxonomía que está evolucionando rápidamente de los picornavirus entéricos aviares. Se llevó a cabo la presente investigación para producir un análisis comparativo de la metagenómica del viroma intestinal de una parvada sana contra una parvada colocada en el campo. Esta investigación reveló una serie de secuencias de picornavirus entéricos que estaban presentes en las aves comerciales en el campo y que estaban completamente ausentes en la parvada sana. Un nuevo ensayo de diagnóstico molecular se utiliza para seguir la diseminación de cepas de campo de picornavirus entéricos en pavipollos comerciales inoculados con homogeneizados intestinales positivos a la presencia de picornavirus preparados a partir de pavos que estaban experimentando enfermedades entéricas moderadas. La propagación de estos nuevos picornavirus entéricos en pavipollos comerciales resultó en una reducción significativa en la ganancia de peso y sugiere que este habitante común del intestino del pavo puede dar lugar a problemas de rendimiento o enfermedad entérica en el campo.

Key words: turkey, enteric, picornavirus, metagenomics

Abbreviations: cDNA = complementary DNA; DPI = days postinoculation; PBS = phosphate-buffered saline; RT-PCR = reverse-transcriptase polymerase chain reaction; SPF = specific pathogen free (SPF); TAU = teaching area unit; TCoV = turkey enteric coronavirus

Our previous research into the dynamic viral community in the turkey gastrointestinal tract revealed a number of novel enteric viruses (6,9). Of particular note in this previous metagenomic investigation was the observation of a number of novel avian enteric picornaviruses, in addition to numerous other known, novel, and minimally described RNA viruses. A follow-up investigation to characterize the novel turkey enteric picobirnaviruses initially described during this metagenomic analysis supported the utility of this approach to identify unknown viruses in the poultry gut (10). A similar approach utilizing the isolation of viral particle-associated nucleic acid from turkey and chicken gut contents led to the complete genomic characterization of novel poultry parvoviruses and subsequent investigations linking the poultry parvoviruses with enteric disease (11,23,24). It is evident that the poultry gut viral community remains only partially described and is incompletely understood. Subsequent investigations into the microbiome of the poultry gut have added to the knowledge regarding the geographical distribution and the rapidly evolving taxonomy of the avian enteric picornaviruses (2,3,12,18,22). The present investigation was undertaken to produce a comparative metagenomic analysis of the gut virome from a healthy turkey flock versus a flock placed in the field. This investigation revealed a number of enteric picornavirus sequences that were present in the commercial birds in the field—the birds in the commercial flock did not perform as well as the healthy flock—that were completely absent in the healthy flock. Further, an RT-PCR assay was developed targeting the turkey enteric picornavirus 3D pol sequence. This assay was used to track the shedding of field strains of turkey enteric picornavirus in commercial poults inoculated with picornavirus-positive intestinal homogenates prepared from turkeys (Arkansas, United States) that were experiencing moderate enteric disease. Our attempt to propagate this novel enteric picornavirus in commercial poults resulted in significant reduction in weight gain over the course of the experiment, and suggests that this common inhabitant of the turkey gut may result in performance problems and enteric disease in the field. Further, the avian enteric picornaviruses have proven to be difficult to propagate in cell culture or in embryonated chicken eggs, although in our hands the avian picornaviruses from turkeys in the southeast United States appear to grow at low levels in specific-pathogen–free (SPF) embryonated turkey eggs.

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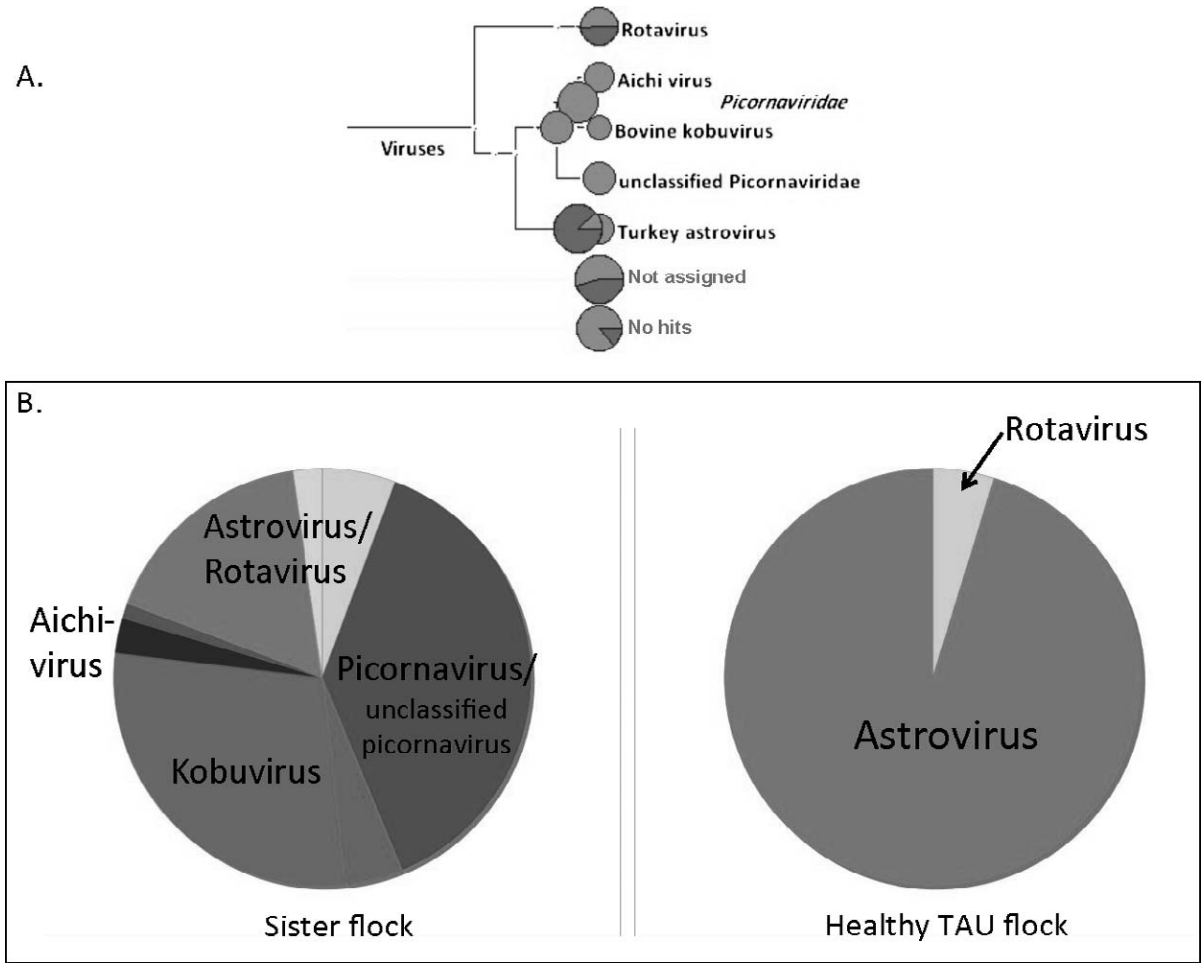

Fig. 1. (A) Taxon tree based upon blastx hits prepared in MEGAN comparing the gut virome present in healthy (dark gray) and affected (light gray) turkey flocks. (B) Graphs representing the viral taxa in the healthy flock versus the sister flock, indicating the proportion of total viral reads belonging to each viral taxa in each flock.

MATERIALS AND METHODS

Comparative metagenomic analysis and reverse-transcriptase PCR (RT-PCR) for enteric picornavirus. A comparative metagenomic analysis of the RNA virus communities present in a teaching area unit (TAU) flock raised under high biosecurity at North Carolina State University and a sister flock comprised of poults from the same source hatchery but placed on a commercial farm in the southeastern United States was performed as previously described (6), with some modifications. Specifically, following the isolation of the enteric viral RNA and the generation of complementary DNA (cDNA), the respective cDNA libraries were labeled with molecular identifier tags (DNA bar coding) that allowed the libraries to be multiplexed on a single picoliter pyrosequencing plate array (454 Biosciences/Roche GS-FLX platform). The respective sequence files (TAU versus commercial) were demultiplexed and assembled into contigs with the use of the gsAssembler software (454 Life Sciences); contigs were used to search the BLAST nonredundant protein database (GenBank) with the use of the blastx program. Blastx hits were analyzed with the use of MEGAN for assignment of contigs to viral taxa and the generation of a comparative taxon tree (13,14). Based upon the contigs derived from the comparative metagenomic analysis and the number of avian enteric picornavirus sequences available in the public databases, an RT-PCR assay directed against the turkey enteric picornavirus polymerase (3D pol; RNA-dependent RNA polymerase gene) was designed. This assay included the forward primer *trk picorna fwd 1* (5′-CTT CTG TGT GTT TTA ATC-3′) and the reverse primer *trk picorna rev 1* (5′-CAA ACA CTC ATA CAA GTT TG-3′). The assay included an RT incubation of 50 C for 30 min followed by 95 C for 15 min; PCR cycling consisted of 35 cycles of 94 C for 30 sec, 48 C for 30 sec, and 72 C for 1 min, followed by a final incubation at 72 C for 10 min. This RT-PCR assay produced an amplicon of 1012 bp; RT-PCR products were resolved on a 1.0% agarose gel. PCR follow-up reactions were performed on purified RT-PCR reactions (Qiagen Qiaquick PCR purification kit) with the use of Pfu polymerase and the same cycle conditions used for the RT-PCR (New England Biolabs Phusion high fidelity PCR kit).

Receipt and handling of field samples. Frozen whole intestinal samples from TAU and commercial flocks were shipped overnight on wet ice and were received at Southeast Poultry Research Laboratory (SEPRL/USDA/ARS, Athens, GA). Following preparation of ~20% intestinal homogenates in phosphate-buffered saline (PBS), the samples were either used directly for RNA isolation as previously described (5,7,20), or the homogenates were stored at −80 C for later use. Purified RNA from the Arkansas field samples was used to assay for turkey enteric coronavirus (TCoV) via real-time RT-PCR as described previously (7,21). Further, these enteric samples were assayed for the presence of avian enteric reovirus, rotavirus, and astrovirus as described previously (8,19). Arkansas field samples were also assayed for the presence of enteric picornavirus with the use of the assay described above.

Inoculation of homogenates into experimental poults and propagation of enteric picornaviruses in embryonated turkey eggs. Twenty percent intestinal homogenates were clarified via centrifugation (2400 × *g*) for 10 min at 4 C and were then filtered with the use of a

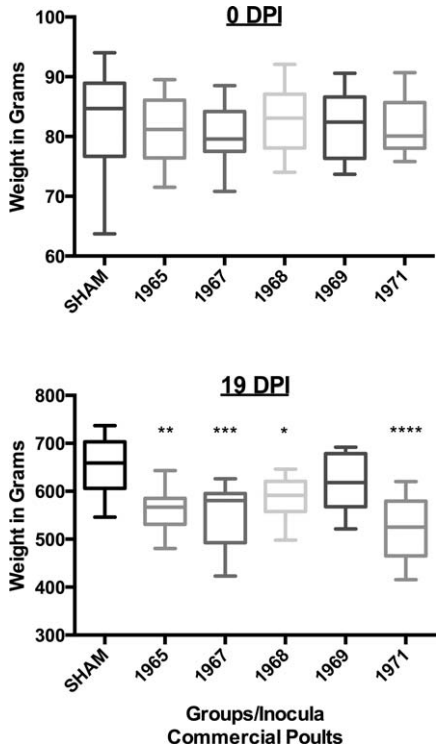

Fig. 2. Graph indicating the body-weight comparisons in experimental and control (SHAM) birds at 0 and 19 DPI. Experimental birds were inoculated with enteric homogenates as described in the text. Asterisks indicate statistically significant reductions in body weights at 19 DPI. Whiskers indicate the minimum and maximum weights in each group (range), and the middle bar indicates the median weight for each group.

0.45-μm syringe filter (Nalgene) and held on ice. Five treatment inocula designated 1965, 1967, 1968, 1969, and 1971 were prepared in this manner. Day-old commercial poults were provided by an industry stakeholder; the poults were divided into six groups of 15 birds and were housed in isolators with *ad libitum* access to feed and water. Prior to placement in the isolators, six birds were swabbed to collect preinoculation fecal samples. Two days following placement, at 0 days postinoculation (DPI), birds were weighed and 250 μl of prepared inoculum was administered to each bird via oral gavage; one SHAM inoculated control group received 250 μl of PBS. Birds were weighed during the course of the experiment at 6, 9, 13, 16, and 19 DPI. Cloacal swabs were collected from at least 10 poults in each group at 2, 6, 9, 13, and 19 DPI; swabs were placed in brain-heart infusion broth and were held on ice and then placed at −80 C until being processed. This experiment was conducted following the guidelines set forth by the SEPRL Institutional Animal Care and Use Committee. For embryonated turkey egg propagation, picornavirus-positive swab material was pooled from groups receiving the 1965, 1967, 1968, and 1971 inocula and was clarified for 10 min at 4 C and 2400 × *g* and filtered (0.45-μm syringe filter as above). Cell-culture antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B) plus 50 μg/ml gentamycin (final concentrations) were added to the clarified and filtered swab material and incubated for 1 hour at room temperature. SPF embryonated turkey eggs (18 days incubation) were inoculated with 200 μl of prepared swab material via the yolk sac route and incubated out to 21 or 24 days, at which time embryo intestines were harvested. Embryo intestines were processed in the same manner as intestinal field samples prior to RNA extraction. RNA extracted from experimental cloacal swabs and from embryo intestines was used in the enteric picornavirus RT-PCR assay as described above.

RESULTS AND DISCUSSION

Comparative metagenomic analysis and RT-PCR for enteric picornavirus. Based upon compelling results from a previous metagenomic investigation of the turkey gut RNA virus community (6), a comparative viral metagenomic analysis was undertaken to compare the RNA virus community in the intestines of the 2010 North Carolina State Teaching Area Unit flock and turkeys and a ''sister'' flock placed in the field. The poults in the TAU flock and the sister flock were obtained from the same hatchery source. Qualitative indicators of enteritis (diarrhea, unevenness) were noted in the sister flock and the overall feed conversion was poorer in the sister flock as well (2.07 versus 1.71 in the TAU flock). The cDNA produced from the ''healthy'' and ''affected'' flocks was assembled into contigs and compared with the use of MEGAN, resulting in a normalized comparative taxon tree and comparison charts (Fig. 1A,B). This comparison revealed that the healthy flock did have apparent infections with turkey astrovirus and rotavirus that were also present in the commercial flock. The commercial flock had numerous contigs with homology to member of the *Picornaviridae* family of viruses that were absent in the healthy flock. In our previous metagenomic analysis of the turkey RNA virome, members of the *Picornavirales* Order dominated the virus community present in the pooled intestinal contents from turkeys on farms in North Carolina (6). The enteric picornaviruses in turkeys in the southeast United States appear to be similar to the avian turdiviruses and the avian galliviruses described elsewhere (2,3,9,22). The nomenclature of the enteric picornaviruses observed in poultry and other avian species is presently in flux and evolving rapidly (1,18), but it is evident that the poultry gut and perhaps the general environment of poultry houses and production areas serves as a reservoir for avian enteric picornaviruses in general. Based upon this comparative analysis and upon the increasing amount of avian picornavirus sequence data available in public databases, we designed an RT-PCR assay targeting the turkey enteric picornavirus 3 D pol gene (the picornaviral polymerase). This RT-PCR assay has been used to confirm the presence of turkey enteric picornavirus in archived enteric field samples held at SEPRL in Athens (data not shown).

Inoculation of homogenates into experimental poults and propagation of enteric picornaviruses in embryonated turkey eggs. Recently, isolated turkey flocks in Arkansas in areas that have

Table 1. RT-PCR assay results for enteric picornavirus.

	Cloacal swabs (+/total)				
Group	2 DPI	6 DPI	9 DPI	13 DPI	19 DPI
SHAM	0/15	0/10	0/10	0/10	0/10
1965	9/11	10/10	9/10	5/10	7/10
1967	11/11	10/10	9/10	4/10	6/10
1968	5/15	10/11	9/11	9/10	10/10
1969	0/11	1/12	0/10	0/10	0/10
1971	12/12	10/12	10/10	4/10	4/10

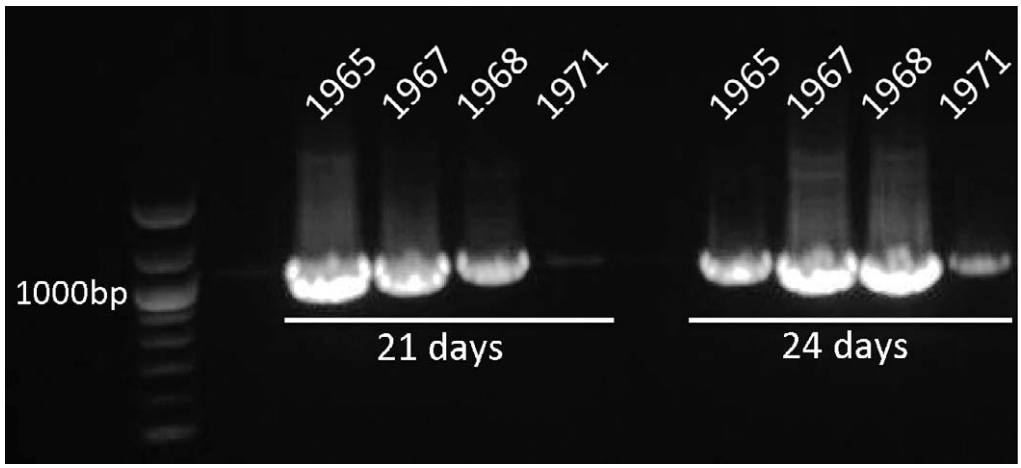

Fig. 3. Results of PCR follow-up assay for the presence of enteric picornavirus in embryo intestines following inoculation of embryonated turkey eggs at 18 days and harvest at 21 and 24 days incubation. One percent agarose gel, SYBer green stain.

experienced ongoing moderate to severe outbreaks of TCoV (7) presented with enteric disease problems that did not follow the observed progression of TCoV infection. Intestinal samples collected from these flocks were sent to our laboratory to confirm the absence of TCoV and to assay for other enteric viruses. The six enteric samples were confirmed as negative for TCoV with the use of real-time RT-PCR (21); the six samples were also found to be negative for the presence of the common poultry enteric pathogens rotavirus and reovirus, and four of the six had evidence of infections with turkey enteric astrovirus (data not shown). Four of the six samples were confirmed positive for turkey enteric picornavirus with the use of the RT-PCR assay reported here. In an attempt to propagate this novel virus, five of the six field homogenates were inoculated into commercial poults at 3 days of age. Over the course of the 3-wk experiment, most poults receiving the intestinal homogenate inocula showed significant reductions in body weight compared to the control birds. Group 1968 initially did not show statistically significant weight reduction through 16 DPI, but had significant reduction in body weights by 19 DPI ($P = 0.004$; Fig. 2). The group receiving the inoculum that was confirmed picornavirus- and astrovirus-free via RT-PCR did not have significant reduction in body weight at any point during the experiment (group/inoculum 1969; Fig. 2). The four experimental groups receiving the field homogenates shed picornavirus at some level throughout the experiment, whereas group 1969, which received the picornavirus-free inoculum, did not (with the exception of one picornavirus-positive swab at 6 DPI; Table 1). Groups 1965, 1967, 1968, and 1971 were shedding turkey astrovirus at 2 DPI, whereas groups 1969 was not; group 1967 had only 1 swab out of 10 positive for turkey astrovirus, however (data not shown). It will be important as the avian enteric picornaviruses are further characterized to obtain pure picornaviral cultures, an outcome that is becoming markedly difficult given the large number of concomitant viral infections that appear to be common in the poultry gut. The current analysis cannot discount the effect turkey astrovirus may have on the noted body weights, but the astrovirus infection did appear to be at low levels in at least one affected group (1967, $P = 0.0005$).

Although the attempt to propagate the enteric picornaviruses in poults was successful, the results in embryonated SPF turkey eggs were mixed. Initially, RNA extracted from the intestines of embryos inoculated at 18 days incubation and harvested at 21 and 24 days of incubation were negative for the presence of picornavirus with the use of our RT-PCR assay. However, after cleaning up the RT-PCR reaction and including a portion in a follow-up PCR assay with high-fidelity DNA polymerase, each egg-passaged sample was positive for enteric picornavirus at both the 21- and 24-day time points (Fig. 3). No attempt was made to perform multiple blind passages in embryonated eggs. This result does indicate that in addition to the propagation of these enteric picornaviruses in young poults, the use of embryonated eggs may be an option. The use of multiple passages in turkey eggs may be warranted in order to establish an *in ovo* propagation protocol for these viruses. Other attempts to propagate the avian enteric picornaviruses in cell culture and in embryonated chicken eggs have not been successful (18).

This comparative metagenomic analysis of the gut virome in turkeys raised under different conditions identified enteric picornaviruses that may be involved in enteric disease and performance problems commonly noted in the field. The present analysis documents the use of the sequence data obtained in that comparative analysis to design a targeted molecular diagnostic assay specifically for the turkey enteric picornaviruses. The propagation of enteric picornavirus in commercial turkeys and embryonated eggs was investigated and compared as well. The fact that we have identified the turkey enteric picornaviruses in different geographic locations and over the course of several years suggests these viruses may be widespread in poultry. The RT-PCR assay described here will facilitate future surveys for these novel picornaviruses in turkeys in the United States. Much work remains to be done to characterize the avian enteric picornaviruses fully, and multiple strains of the viruses may be present concomitantly in the poultry gut (18). Enteric diseases with suspected viral etiology such as poult enteritis complex and poult enteritis syndrome are ongoing problems in the United States turkey industry, in addition to enteric diseases of known viral etiology such as turkey coronavirus enteritis (7,15–17,19,20). These investigations are often difficult, because the suspected viruses are often also found in healthy birds and because of the difficulty of reproducing the full syndromes with isolated viruses. Further, regional performance problems such as light turkey syndrome in the upper midwest United States appear to have underlying bacterial causes (4). This sort of investigation of the dynamic gut microbial community will provide the tools necessary to uncover the overlapping roles viruses, bacteria and other intestinal pathogens play in performance problems and acute intestinal disease in poultry.

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