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Source: Avian Diseases, 49(1):86-91. 2005.
Published By: American Association of Avian Pathologists
DOI: http://dx.doi.org/10.1637/7265-082304R
URL: http://www.bioone.org/doi/full/10.1637/7265-082304R

AVIAN DISEASES 49:86–91, 2005

Multiplex Real-Time Reverse Transcription–Polymerase Chain Reaction for the Detection of Three Viruses Associated with Poult Enteritis Complex: Turkey Astrovirus, Turkey Coronavirus, and Turkey Reovirus

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Received 23 August 2004; Accepted 7 October 2004

SUMMARY. Poult enteritis complex (PEC) is an economically important disease of young turkeys characterized by diarrhea, poor weight gain, and, in some cases, high mortality. Although PEC is considered to be a polymicrobial disease, numerous viruses, including turkey coronavirus (TCV), turkey astrovirus type 2 (TAstV-2), and avian reoviruses (ARVs), have been associated with PEC-like disease. Real-time reverse transcription–polymerase chain reaction (RRT-PCR), a highly sensitive and specific detection method for viral RNA, was developed in a multiplex format for the simultaneous detection of TAstV-2 and TCV and for the detection of two genetic types of ARV. Assay sensitivity was determined using *in vitro* transcribed RNA and varied by target between 150 gene copies for TAstV-2 alone and 2200 gene copies for TCV when multiplexed. Virus detection was evaluated with samples collected from poults inoculated at 1 day of age with each of the viruses. Cloacal swabs and intestinal samples were obtained at 1, 2, 3, 4, 6, 9, 14, 17, and 21 days after inoculation, processed, and tested for virus detection by RRT-PCR. Cloacal swabs from TAstV-2– and TCV-infected poults were shown to have sensitivity for virus detection similar to that of intestinal samples when compared directly. ARV detection by RRT-PCR was compared with virus isolation and had similar sensitivity.

RESUMEN. Prueba múltiple de la transcriptasa reversa-reacción en cadena por la polimerasa en tiempo real para la detección de tres virus asociados con el complejo de enteritis en pavipollos: astrovirus, coronavirus y reovirus del pavo.

El complejo de enteritis de los pavipollos es una enfermedad económicamente importante caracterizada por diarrea, baja ganancia de peso, y en algunos casos, mortalidad elevada. Aunque el complejo de enteritis de los pavipollos es considerado como una enfermedad multimicrobiana, varios virus, incluyendo el coronavirus de los pavos, el astrovirus tipo 2 de los pavos y los reovirus aviares, han sido asociados con una enfermedad similar a la del complejo de enteritis de los pavipollos. La prueba de la transcriptasa reversa-reacción en cadena por la polimerasa en tiempo real es un método de alta sensibilidad y especificidad para la detección del ARN viral. Se desarrolló una prueba de formato múltiple para la detección simultánea del astrovirus tipo 2 y el coronavirus de los pavos, y la detección de dos tipos genéticos de reovirus aviar. Se determinó la sensibilidad de la prueba empleando plantillas de RNA trascritas *in vitro*, mostrando una variación de acuerdo a la plantilla empleada de 150 copias de genes para el astrovirus del pavo tipo 2 y de 2200 copias para el coronavirus de los pavos, al ser empleadas simultáneamente. Se evaluó la detección de los virus empleando muestras tomadas de pavipollos inoculados al día de edad con cada uno de los virus. Se tomaron y procesaron muestras de hisopos cloacales e intestinales a los días 1, 2, 3, 4, 6, 9, 14, 17, y 21 posteriores a la inoculación para determinar la presencia de los virus mediante la prueba múltiple de la transcriptasa reversa-reacción en cadena por la polimerasa en tiempo real. Se observó una sensibilidad similar en la detección viral a partir de los hisopos cloacales provenientes de pavipollos infectados con el astrovirus tipo 2 o con el coronavirus de los pavos y las muestras intestinales correspondientes al ser comparadas directamente. Se observó una sensibilidad similar en la detección de reovirus aviares mediante la prueba múltiple de la transcriptasa reversa-reacción en cadena por la polimerasa en tiempo real y el aislamiento viral.

Key words: poult enteritis, turkey coronavirus, turkey astrovirus, avian reovirus, real-time reverse transcription–polymerase chain reaction

Abbreviations: ARV = avian reovirus; EM = electron microscopy; IBV = infectious bronchitis virus; PEC = poult enteritis complex; RRT-PCR = real-time reverse transcription–polymerase chain reaction; RT-PCR = reverse transcription–polymerase chain reaction; SEPRL = Southeast Poultry Research Laboratory; SPF = specific-pathogen-free; TAstV-2 = turkey astrovirus type 2; $TCID_{50}$ = 50% tissue culture infectious doses; TCV = turkey coronavirus; VI = virus isolation

Poult enteritis complex (PEC) is a disease condition of turkeys younger than 6 wk. The primary disease presentation of PEC is diarrhea, restlessness, and a general poor condition of the poult (2). In cases in which mortality is high, the disease may be classified as poult enteritis mortality syndrome (1). PEC causes substantial economic losses in the turkey industry, as the full genetic growth potential of the turkey cannot be achieved because of stunting and decreased weight gain. PEC is a major concern in the southeastern United States, where it is estimated that 60%–90% of all flocks may experience PEC-related disease during the spring and summer (1). Similar disease conditions have been reported from most regions where turkeys are commercially produced (1). Although the etiologic agent (or agents) that causes PEC has not been definitively identified, several viruses have been associated with PEC-like disease, including turkey astrovirus type 2 (TAstV-2) (9,19), turkey coronavirus (TCV) (6,22), and avian reoviruses (ARVs) (7).

Current methods for detection of these viruses in clinical cases of PEC include electron microscopy (EM), immune EM, antigen capture enzyme-linked immunosorbent assay and fluorescent antibody techniques, standard reverse transcription–polymerase chain reaction (RT-PCR)–based methods (1,3,4,5,10,11,12), and, to a limited extent, virus isolation (VI), because not all of the agents are culturable. Most of these techniques lack the high sensitivity and high specificity that can be achieved with real-time RT-PCR (RRT-PCR).

Table 1. Sequences of RRT-PCR primers and probes.

Virus target	Target gene	Name	Sequence
TAstV-2	Polymerase	TAV 4248F	5′-TCC TCC ATG ATT CTC ATA AG -′3
		TAV 4360R	5′-CTT GAC CTG GCA AAC T-′3
		TAV 4274PB	5′-{FAM}-AAG ATG CGG CGC TTG TA- {TAMRA}-′3
TCV	Matrix	TCV 2F	5′-AGT GGC TTG CTA AGT G -′3
		TCV 112R	5′-GCT TTG GTC ACC AGT -′3
		TCV 51PB	5′-{TXRed}-TAT GCA CAC CGG ATA GAC G-{BHQ-2}-′3
Chicken-origin reovirus	S1	S1 8F	5′-GTC TCA ATC CAT CGC A-′3
		S1 110R	5′-AGC CGT TCA TAG ATC G-′3
		S1 35PB	5′-{FAM}-TCG TCA GCT TGA TAC TGT CAT T-{BHQ-1}-′3
Turkey-origin reovirus	S3	S3 122F	5′-ATG TGA TCA AGG TCG GTA A-′3
		S3 230R	5′-GGT GAC ACT TGT GGT-′3
		S3 147PB	5′-{TX Red}-TTG TTG CTC TCA ATG CTG-{BHQ-2}-′3

RRT-PCR amplifies viral nucleic acid in a manner similar to standard RT-PCR; however, the product is detected in real time with a sequence-specific probe labeled with fluorescent dyes (14). RRT-PCR has the additional advantage of being well suited to multiplexing, as each target can be differentiated because the probes can be labeled with dyes that fluoresce at different wavelengths and can therefore be recognized as separate signals by the RRT-PCR instrument. Although the cost of RRT-PCR is close to that of standard RT-PCR (17), multiplexing reduces costs and time by simultaneously testing for several agents. Real-time PCR methods are also faster, more specific, and less prone to contamination than standard PCR (15,16).

Two multiplex RRT-PCR tests were developed, one test for the detection of TAstV-2 and TCV and a second test for the detection of ARV. The ARV test targets two different genes on separate segments of the reovirus genome to detect as broad a range of ARVs as possible. Test sensitivity was determined for each target virus with *in vitro* transcribed RNA. The ability of the test to detect each virus in tissues routinely sampled for these viruses was evaluated with cloacal swabs and intestinal samples collected at regular intervals from poults inoculated at 1 day of age with each of the viruses.

MATERIALS AND METHODS

Viruses. The TAstV-2 reference isolate was NC/96 (9,19). RNA from additional TAstV-2 isolates was obtained from commercial turkeys reared in the southeastern United States. These isolates were confirmed to be TAstV-2 by sequencing. The TCV reference isolate VA/SEP-C26/03 was collected from commercial turkeys in the southeastern United States and was passaged once in turkey embryos. Five additional TCV isolates collected between 1996 and 2003 in the southeastern United States from the Southeast Poultry Research Laboratory (SEPRL) repository were used for TCV specificity testing. The identity of the viruses was confirmed by sequencing. Two reference isolates were used for ARV, one to represent each genotype: S1133 (21) represented chicken-origin reoviruses and NC/98 (8) represented turkey-origin reoviruses. Two additional chicken-origin reoviruses, 1733 and MissB (18), were provided by Dr. John Rosenberger (University of Delaware, Newark, DE). Additional turkey-origin reoviruses used to demonstrate specificity included NC/PEMS/85, TX/98, TX/99, and ATCC-C (20) and three other recent isolates from commercial turkeys in the southeastern United States, NC/SEP-R44/03, NC/SEP-R61/03, and NC/SEP-R108/03.

RNA extraction. RNA was extracted from 250 μl of cloacal swab material with Trizol LS reagent (Invitrogen, Inc., Carlsbad, CA) in accordance with the manufacturer's instructions. RNA was extracted from intestinal tissue by flushing the contents with sterile phosphate-buffered saline (20% w/v). Up to 1 ml of the wash material was removed and centrifuged for 15 min at 14,000 × *g*. The supernatant (250 μl) was used for RNA extraction as described for the cloacal swab material.

Multiplex RRT-PCR. Two multiplex RRT-PCR assays were developed and optimized: TAstV-2/TCV and ARV (which targets the S1 gene segment of chicken-origin reoviruses and the S3 gene segment of turkey-origin reoviruses). Primer and probe sequences are given in Table 1. RRT-PCR was run on a Cepheid Smart Cycler (Cepheid Inc., Sunnyvale, CA) in a 25-μl volume with the Qiagen OneStep RT-PCR kit (Qiagen Inc., Valencia, CA). The reverse transcription step was the same for all tests: 50 C for 30 min and 95 C for 15 min. The TAstV-2/TCV reaction conditions were as follows: 1× Qiagen OneStep RT-PCR kit reaction buffer, 320 mM deoxyribonucleotide triphosphate mix, 3.75 mM magnesium chloride, 10 pmol of each primer (except for TCV 2F, which used 20 pmol per reaction), 0.1 μM TAstV-2 probe, 0.2 μM TCV probe, and 1 μl of Qiagen RT-PCR enzyme blend. The ARV-S1-S3 reaction conditions were as follows: 1× Qiagen OneStep RT-PCR kit reaction buffer, 320 mM deoxyribonucleotide triphosphate mix, 3.75 mM magnesium chloride, 10 pmol of each primer, 0.1 μM ARV S1 probe, 0.2 μM ARV S3 probe, and 1 μl of Qiagen OneStep RT-PCR enzyme blend.

Sensitivity and specificity. RNA for determining assay analytic sensitivity and for use as positive controls was produced by *in vitro* transcription of a target template containing a 1000-base pair portion of each RRT-PCR target gene sequence that was produced by RT-PCR with primers containing the T7 promoter sequence. The transcription reactions were performed with the Promega RiboMax T7 kit (Promega, Madison, WI) in accordance with the manufacturer's instructions. After digestion of template DNA with DNase (Ambion Inc., Austin, TX), RNA was purified by extraction with Trizol LS reagent (Invitrogen, Inc.) and quantified by ultraviolet spectrophotometry. To determine the minimum amount of RNA each test could detect regardless of sample type or RNA extraction procedure, 10-fold and 2-fold dilution series of *in vitro* transcribed RNA, each run in triplicate at least, were used to determine the gene copy limits of detection for each test in both the single reaction and multiplex formats.

The specificity of each assay was evaluated by testing all four target reference viruses with all four RRT-PCR tests. In addition, RNA samples isolated from five TCV isolates was tested. The TCV test was also evaluated for cross-reactivity with RNA isolated from infectious bronchitis virus (IBV) (Mass type), a common avian coronavirus in chickens. The TAstV-2 test was evaluated with RNA from the NC/96 isolate, which was considered the reference isolate, and five additional recent field isolates. The specificity of the ARV-S1-S3 test was evaluated with RNA from 10 additional isolates as already described.

Samples from experimentally inoculated poults. Specific-pathogen-free (SPF) turkey poults were obtained at 1 day of age from SEPRL flocks and divided into four groups of 20 each and one group of 10 that served as sham-inoculated controls. Each group of 20 poults was inoculated by the oral route with a sterile oral gavage needle with 0.2 ml of one of the following: TAstV-2 NC/96 isolate, $10^{3.5}$ 50% tissue

culture infectious doses ($TCID_{50}$) of ARV NC/98 isolate, $10^{3.5}$ $TCID_{50}$ of ARV S1133 isolate, or TCV VA/SEP-C26/03 isolate. Controls were sham inoculated with 0.2 ml of sterile phosphate-buffered saline. Poults were housed in Horsfall isolators with *ad libitum* access to food and water. Poults were observed daily for clinical signs. Dead birds were necropsied and gross lesions recorded.

Cloacal swabs were collected in sterile brain-heart infusion broth (Becton Dickinson, Sparks, MD) at 1, 2, 3, 4, 6, 9, 14, 17, and 21 days after inoculation from 10 poults per group until day 14, after which only five swabs were obtained per group. Two birds from each group were euthanatized and necropsied at 2, 4, 6, 9, 14, and 21 days after inoculation, at which time intestinal samples (jejunum and ceca) were collected for virus detection by RRT-PCR. RRT-PCR results for TAstV-2 were confirmed by standard RT-PCR (10) on selected positive and negative samples.

Samples from experimentally inoculated chickens. One-day-old SPF White-Rock chickens obtained from SEPRL flocks were divided into groups of 10 chicks and inoculated with $10^{3.5}TCID_{50}$ of the 1733 ARV isolate by the oral route. Cloacal swabs and intestinal tissues (pool of all intestines) were collected at 2, 4, and 7 days after inoculation. Tissue and swab samples were processed for ARV-S1-S3 RRT-PCR and VI as described for experimentally infected poults.

ARV VI. VI in Vero cells was used as the reference detection method for ARV for both swabs and tissue samples. ARV was isolated from cloacal swabs by centrifuging 500 μl of swab material for 15 min at $14{,}000 \times g$. Supernatant was removed and incubated with 10,000 IU/ml of penicillin, 2,000 μg/ml of streptomycin, and 20 μg/ml of amphotericin B (Mediatech, Inc., Herndon, VA) for 30 min at room temperature.

Vero cells were grown in 24-well cell culture plates in 50%F12/50% Dulbecco modified Eagle medium (Mediatech, Inc.), 8% fetal bovine serum (Mediatech, Inc.), and 100 IU/ml of penicillin, 100 μg/ml of streptomycin, 0.25 μg/ml of amphotericin B (Mediatech, Inc.) until a monolayer density of between 70% and 80% confluence was achieved, at which time the media were removed and 100 μl of processed sample was inoculated. Each sample was tested in duplicate. Plates were incubated for 30–60 min to allow the inoculum to adsorb, then maintenance media (2% fetal bovine serum) were added and the plates were incubated for 5–6 days at 37 C and 5% carbon dioxide and observed daily for cytopathic effects. Wells exhibiting cytopathic effects were considered positive, and selected samples were confirmed to be ARV-positive by RRT-PCR.

Samples that were VI negative and RRT-PCR positive were confirmed to be positive by standard RT-PCR with the following primers for S1133 samples: S1 8F (Table 1) and S1 867R 5′-GTACGCATGAGTCGCAG-3′. Primers for NC/98 samples were S3 1F 5′-ATGGAGGTACGTGTGC-3′ and S3 918R 5′-GACTTTGAC-CACCCACG-3′. The Qiagen OneStep RT-PCR kit was used in accord with the manufacturer's instructions. RT-PCR products were visualized on 1% agarose gel, and selected positive samples were excised and extracted with the Qiagen gel extraction kit. Direct sequencing was performed on the extracted RT-PCR products with the BigDye terminator kit (Applied Biosystems, Foster City, CA) and were subsequently run on an ABI 3730 (Applied Biosystems). Sequences were analyzed by alignment with the appropriate genes from the inoculated virus by ClustalW (Lasergene, DNAStar, Madison, WI) or by BLAST search against sequences in GenBank.

Statistical analysis. The correlation of results obtained by RRT-PCR with cloacal swabs *vs.* intestinal tissue pools was determined with McNemar test at $P < 0.05$ (SigmaStat 3.0 software; Systat Software, Inc., Richmond, CA). McNemar test ($P < 0.05$) was also used to determine the correlation of RRT-PCR results with VI for detection of ARV.

RESULTS

Sensitivity. Limits of detection were determined with *in vitro* transcribed RNA for each target template in both the single reaction and multiplex reaction formats (Fig. 1). Detection limits varied between 150 and 2200 gene copies by target (Table 2). Multiplexing affected the sensitivity in the TAstV-2/TCV test, in which the detection limit increased from approximately 150 to 600 copies for TAstV-2 and from 1100 to 2200 copies for TCV. The ARV-S1-S3 test had a limit of detection of 1400 to 2000 copies and approximately $10^{1.6}$ $TCID_{50}$ compared with VI.

Specificity. The TAstV-2/TCV test was able to detect the NC/96 isolate and the five additional recent field isolates as TAstV-2. All five TCV isolates tested produced a positive result for the TCV component of the TAstV-2/TCV RRT-PCR test. IBV RNA also produced a positive result with the TCV test. All ARVs tested were detected with either the S1 or S3 component of the ARV-S1-S3 multiplex test (Table 3). Two isolates (NC/PEMS/85 and TX/98) could be detected with both the S1- and S3-specific components.

Detection of virus in experimentally inoculated animals by RRT-PCR. In poults experimentally inoculated with TAstV-2, virus could be detected in both cloacal swabs and intestinal tissue from day 1 to day 14 after inoculation by RRT-PCR (Table 4). The highest proportion of poults was positive for virus detection between days 4 and 9 after inoculation. During all times when virus was detected in intestinal tissue, the corresponding swab material was also positive. Because of mortality, there were no TAstV-2–inoculated poults sampled on day 21 after inoculation. McNemar test at $P < 0.05$ was used to compare virus detection between cloacal swabs and intestinal tissue; for TAstV-2 there was no significant difference ($P = 0.31$).

TCV could be detected in both cloacal swabs and intestinal tissue samples from experimentally infected poults throughout the sampling period. The peak of virus detection was 3–6 days after inoculation, when 100% of cloacal swabs were positive and all intestinal samples were positive except for two of four collected 6 days after inoculation (Table 4). At 6, 9, and 14 days after inoculation, detection of TCV was more consistent in the swabs than from intestinal tissue. Detection of TCV was significantly better in cloacal swabs than in intestinal tissues ($P = 0.01$).

ARV was detected from 2 to 6 days after inoculation in the S1133-inoculated poults and from 1 to 9 days after inoculation in the NC/98-inoculated poults with the S1 and S3 gene probe and primer sets, respectively (Table 4). The 1733 ARV isolate could be detected in all intestinal tissues and swabs from chickens at 2 and 4 days after inoculation (data not shown). At 7 days after inoculation, 1733 was detected in one of two swab samples and in two of two intestinal samples. There was a negative correlation ($P = 0.001$) between cloacal swabs and intestinal tissue for detection of ARVs with either test.

VI in Vero cells was used as a reference method for comparison with RRT-PCR for ARV detection from cloacal swabs and intestinal tissue samples. A total of 142 samples were compared for virus detection by VI and the S1 gene RRT-PCR test, and 99 samples were compared for virus detection by VI and the S3 gene RRT-PCR test. There were 27 samples positive by the S1 gene RRT-PCR test, of which 13 were negative for VI (Table 5). One sample was VI positive and RRT-PCR negative. The S3 gene RRT-PCR test detected 21 positive samples, of which 10 were negative by VI (Table 6). One sample was VI positive and RRT-PCR negative. VI-negative, RRT-PCR–positive samples from both the S1133 and NC/98 groups were confirmed to be positive by standard RT-PCR and subsequent sequencing of selected positive samples. Based on these results, RRT-PCR was significantly more sensitive than VI at $P < 0.05$ using McNemar test ($P = 0.003$ for the S1 gene test and $P = 0.016$ for the S3 gene test). Birds sampled before virus inoculation and sham-inoculated birds were negative for virus detection.

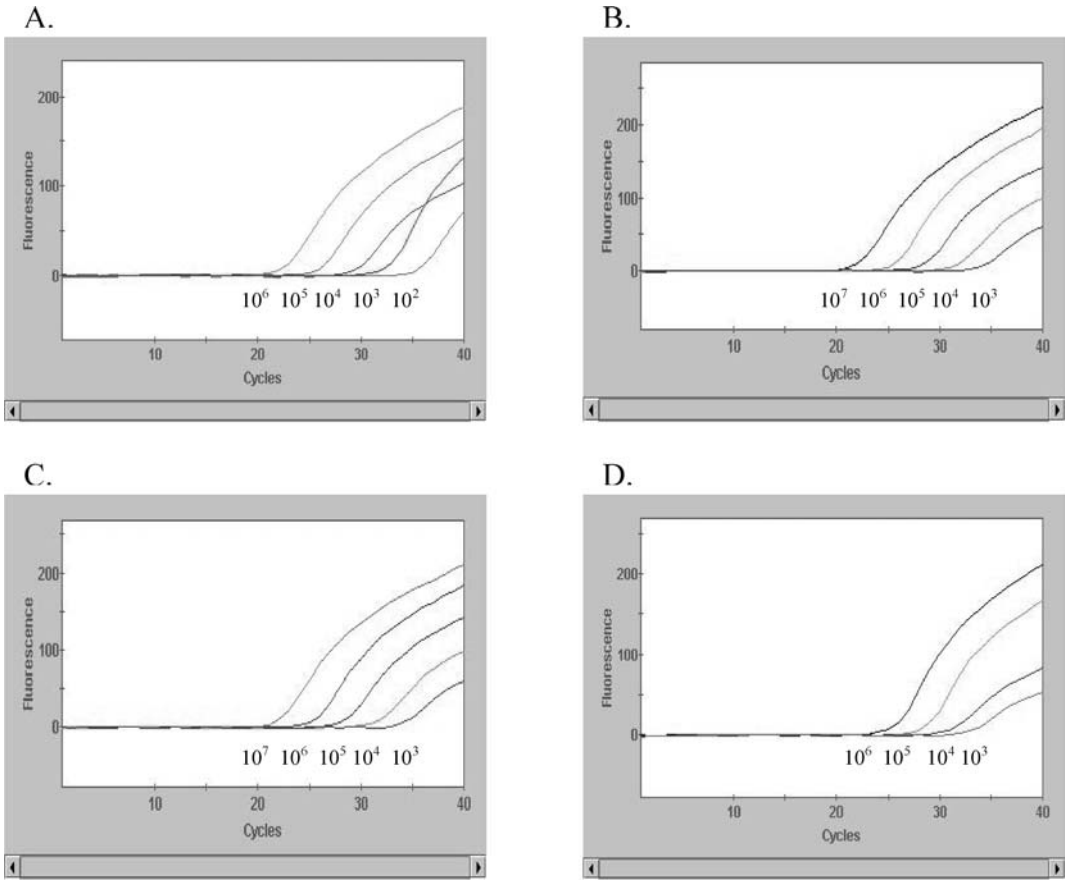

Fig. 1. Smart Cycler fluorographs of 10-fold dilution series of *in vitro* transcribed RNA of each target gene RNA with their respective primer and probe sets run in multiplex. Approximate target RNA copy numbers are shown below each sample curve. (A) TAstV-2. (B) TCV. (C) Chicken-origin reovirus. (D) Turkey-origin reovirus.

Clinical signs and lesions observed. Although an evaluation of the pathogenesis of these viruses was not an objective of this study, the presence of clinical lesions provides evidence that the inoculated poults were infected. Poults inoculated with TAstV-2, TCV, and the NC/98 isolate of ARV experienced diarrhea. TCV-inoculated poults were mildly depressed during the first week after inoculation. Lesions observed in the groups inoculated with TAstV-2, TCV, and NC/98 ARV during necropsy included frothy distended ceca and watery distended intestines during the first week after inoculation. Poults inoculated with the S1133 isolate of ARV displayed mildly distended and watery intestines and ceca. Lesions consistent with PEC were not observed in the sham-inoculated poults.

Table 2. Limits of detection in gene copy numbers for PEC-associated virus RRT-PCR assays as single target tests and when multiplexed (TAstV-2 and TCV are multiplexed, and the chicken-origin reovirus S1 and turkey-origin reovirus S3 gene tests are multiplexed).

Test specificity	Limit of detection (approximate no. of gene copies)	
	Single target reaction	Multiplexed reaction
TAstV-2	150	600
TCV	1100	2200
Chicken-origin reovirus S1 gene	1400	1400
Turkey-origin reovirus S3 gene	2000	2000

DISCUSSION

Current diagnostic methods for PEC-associated viruses, such as EM and fluorescent antibody assays, have limitations because of poor analytic specificity and sensitivity. Consistency of diagnostic test results on samples from turkeys experiencing PEC has been problematic for clinicians and researchers when multiple methods

Table 3. Detection patterns of selected ARV isolates with the chicken-origin reovirus S1 and turkey-origin reovirus S3 gene-specific RRT-PCR assay.

Isolate	Test specificity	
	S1 gene segment	S3 gene segment
Chicken-origin reoviruses		
S1133	+	−
MissB	+	−
1733	+	−
Turkey-origin reoviruses		
NC/98	−	+
NC/PEMS/85	+	+
TX/98	+	+
TX/99	−	+
NC/SEP-R44/03	−	+
NC/SEP-R61/03	−	+
NC/SEP-R108/03	−	+
ATCC-C	−	+

Table 4. Detection of TAstV-2, TCV, chicken-origin reovirus, or turkey-origin reovirus from experimentally inoculated SPF poults by multiplex RRT-PCR by sample type and day after inoculation.

	1 day		2 days		4 days		6 days		9 days		14 days		17 days		21 days	
Treatment group	S[A]	I[B]	S	I	S	I	S	I	S	I	S	I	S	I	S	I
TAstV-2	1/10[C]	nd[D]	3/10	4/4	7/10	2/4	8/10	4/4	4/7	3/4	2/5	1/4	0/3	0/4	nd	nd
TCV	3/10	nd	8/10	1/4	10/10	4/4	10/10	2/4	9/9	0/4	5/5	2/4	4/4	1/3	3/3	3/4
Chicken-origin reovirus	0/10	nd	0/10	2/2	0/10	1/4	0/10	1/3	0/10	0/4	0/5	0/4	0/5	0/4	0/1	0/4
Turkey-origin reovirus	1/10	nd	1/6	2/4	3/10	4/4	3/9	3/4	0/8	1/4	0/6	0/4	0/5	0/4	0/5	0/4

[A]S = swab material (cloacal).
[B]I = intestinal tissue.
[C]Number positive/number tested.
[D]nd = not done.

are used for virus detection. The availability of a consistent method with high sensitivity and high specificity would be beneficial to PEC diagnostics.

Recently, the application of real-time PCR technology to diagnostics has become more widespread in both human and veterinary medicine (16). Real-time PCR–based methods are well suited to diagnostics because of their high sensitivity, high specificity, and inherently quantitative nature (16). They are also rapid and cost-effective on a per-sample basis because of their ability to be multiplexed, a modification in which multiple agents can be targeted for detection in a single reaction. Because of the recent development of multiple diagnostic tests for multiple pathogens from different species, real-time PCR instruments are becoming increasingly more common in veterinary diagnostic laboratories.

In our study, the PEC-associated virus RRT-PCR test exhibited high sensitivity and was able to detect the target viruses from intestinal tissues and cloacal swabs from experimentally infected animals. One objective of this study was to determine whether cloacal swab samples are a suitable sample for the target viruses, as fecal shedding of these viruses has been well established (1,2). Cloacal swab samples are easier to collect, may be pooled from multiple birds, and are easier to process than tissue samples, saving time and money and eliminating the need to euthanatize the birds for sampling.

A positive correlation was observed for RRT-PCR virus detection from cloacal swabs and intestinal tissue for TAstV-2 and TCV. Because this multiplex test is intended for use on a flock basis, the level of virus detection from cloacal swabs compared with intestinal tissue appears to be adequate for TCV and TAstV-2. However, this correlation was not supported for ARVs, in which intestinal tissue was better for sampling.

An ancillary goal of this study was to determine how long each virus was detectable by RRT-PCR in intestinal samples and cloacal swabs collected from poults after exposure at 1 day of age. TAstV-2 was detected in cloacal swabs and intestinal tissues for 2 wk after inoculation. No end point in shedding was reached for TCV, as the virus was still detected 3 wk after inoculation in both swabs and intestinal tissue. A previous report using standard RT-PCR showed TCV detection for up to 49 days after inoculation in birds exposed at 2 wk of age (3). The ARVs were detected for a shorter length of time, up to 6 days after exposure for S1133 and up to 9 days after exposure for NC/98. Because S1133 is a mildly pathogenic chicken-derived ARV strain, it may have limited replication ability in turkeys, which would be supported by the low proportion of positive samples and the short detection period.

Each RRT-PCR test displayed the expected specificity with the available reference isolates. The detection of IBV RNA with the TCV test was expected based on the sequence information available. However, this should not affect the practical application of the test, as IBV is unlikely to replicate in the enteric tracts of turkeys. The test for ARV was designed to target two genes from both chicken- and turkey-origin reoviruses to detect as broad a range of ARVs as possible. Importantly, this test distinction (sequences from chicken-origin and turkey-origin reoviruses) was based on limited nucleotide sequence data that distinguish these viruses (8,20). There are no data to associate the target genes with host specificity or pathogenesis. Further studies are being conducted to determine the clinical importance of these genetically different, turkey-origin reoviruses. Because reoviruses have a segmented genome, reassortment, which is known to occur among reoviruses (13), may account for the isolates (TX/98 and NC/PEMS/85) that react with both the S1 and S3 gene tests.

Because of the lack of a primary reference method for TCV and TAstV-2, RRT-PCR was only compared with VI in Vero cells for ARV. Although the numbers of ARV-positive samples were low (possibly because of poor infection), the RRT-PCR test appeared to be more sensitive than VI, although the limit of detection was $10^{1.6}$ $TCID_{50}$ when compared directly. This discrepancy is most likely because of the difficulty in processing intestinal samples for VI with clinical samples, as the clarification process to prevent contamination may remove virus and debris from the samples may inhibit cell growth or make it difficult to evaluate cytopathic effects.

This report provides an initial bench validation of the TCV, TAstV-2, and ARV multiplex RRT-PCR assays. Based on tissue samples obtained from experimentally infected animals, use of cloacal swabs with the RRT-PCR test would provide an adequate level of

Table 5. Comparison of chicken-origin reovirus detection by VI in Vero cells and by RRT-PCR with the S1-specific primer and probe set. RRT-PCR was significantly more sensitive for ARV detection than VI at $P < 0.05$ ($P = 0.003$).

	RRT-PCR		
VI	Positive	Negative	Total
Positive	14	1	15
Negative	13	114	127
Total	27	115	142

Table 6. Comparison of turkey-origin reovirus detection by VI in Vero cells and by RRT-PCR with the S3-specific primer and probe set. RRT-PCR was significantly more sensitive for ARV detection than VI at $P < 0.05$ ($P = 0.016$).

	RRT-PCR		
VI	Positive	Negative	Total
Positive	11	1	12
Negative	10	77	87
Total	21	78	99

virus detection on a flock basis. The RRT-PCR tests have high analytic sensitivity and specificity, which should provide high consistency in the nucleic acid detection of these viruses in clinical samples.

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ACKNOWLEDGMENTS

This work was supported by USDA CRIS project 6612-32000-020.

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We acknowledge Scott Lee and Dawn Williams-Coplin for technical assistance with the manuscript.