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Development of a Multiplex Reverse Transcription–Polymerase Chain Reaction Diagnostic Test Specific for Turkey Astrovirus and Coronavirus

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SUMMARY. A multiplex reverse transcription–polymerase chain reaction (RT-PCR) assay was developed for the simultaneous detection of two enteric viruses of poultry: turkey enteric coronavirus (TCV) and turkey astrovirus (TAstV). PCR primers were designed to conserved regions within the nucleocapsid gene of TCV and to the polymerase gene of TAstV-2. The primer pairs were successfully used in a multiplex RT-PCR to detect nucleic acid of TAstV-2 and TCV. The test was optimized for use with intestines/feces from naturally infected turkeys. The primers were specific and did not amplify other common RNA or DNA avian viruses. The detection limit was determined to be 10 ng of RNA used as starting template. The use of this specific test allows the rapid and early diagnosis of two financially costly viruses affecting the commercial turkey industry.

RESUMEN. Desarrollo de una prueba diagnóstica múltiple por transcripción reversa y reacción en cadena por la polimerasa específica para astrovirus y coronavirus de los pavos.

Se desarrolló un ensayo múltiple mediante transcripción reversa y reacción en cadena por la polimerasa (de las siglas en Inglés RT-PCR), para la detección simultánea de dos virus entéricos de las aves: el coronavirus entérico de los pavos y el astrovirus de los pavos. Se diseñaron iniciadores específicos para las regiones conservadas dentro del gen de la nucleocápside del coronavirus y del gen que codifica para la polimerasa del astrovirus. Mediante la prueba múltiple de RT-PCR se detectaron exitosamente los ácidos nucleicos de ambos virus utilizando dichos pares de iniciadores. Se establecieron las condiciones óptimas para usar la prueba con muestras de excretas e intestinos de aves infectadas naturalmente. Los iniciadores fueron específicos y no se observó amplificación de otros virus aviares ADN o ARN. Se determinó que el límite de detección de la prueba era de 10 ng de ARN, cantidad que fue utilizada como molde inicial. El uso de esta prueba específica permite el diagnóstico temprano y rápido de dos virus con importancia económica que afectan la producción comercial de pavos.

Key words: multiplex RT-PCR, poult enteritis mortality syndrome, turkey coronavirus, turkey astrovirus, viral diagnostics

Abbreviations: CAV = chicken anemia virus; cELISA = competitive enzyme-linked immunosorbent assay; dNTP = deoxynucleoside triphosphate; EDTA = ethylenediaminetetraacetic acid; EM = electron microscopy; IBDV = infectious bursal disease virus; IBV = infectious bronchitis virus; IFA = immunofluorescence assay; ILTV = infectious laryngotracheitis virus; MMLV = Moloney murine leukemia virus; NDV = Newcastle disease virus; PBS = phosphate-buffered saline; PEMS = poult enteritis mortality syndrome; REO = reovirus; RT-PCR = reverse transcription–polymerase chain reaction; SPF = specific pathogen free; TAE = Tris acetate EDTA; TAstV = turkey astrovirus; TAstV-2 = turkey astrovirus isolate 2; TBE = Tris borate EDTA; TCV = turkey coronavirus; UV = ultraviolet; VI = virus isolation

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Infectious diseases affecting the digestive tract of commercial poultry are proposed to result in more economic loss than those affecting any other system (2). The effects of enteric disease continue long after clinical recovery. A recent example is the outbreak of poult enteritis mortality syndrome (PEMS), a highly infectious disease of young turkeys (3,4,5,8) that has caused devastating financial losses to the turkey industry. Two viruses strongly implicated as etiologic agents in PEMS are turkey enteric coronavirus (TCV) and a novel strain of avian astrovirus (TAsV-2) (6,13,16,17,34).

The avian coronaviruses are members of the *Coronaviridae* family with large, single-stranded, positive sense RNA viruses that have a polyadenylated genome of approximately 30 Kb (19,22). Coronaviruses cause common respiratory and enteric diseases of humans and domestic animals (28,33). In poultry, coronaviruses cause either a respiratory disease in chickens (infectious bronchitis virus, IBV) or an enteric disease in turkeys (TCV) (15). Historically, outbreaks of TCV have been financially devastating to the turkey industry (7,22). Turkeys of any age can be infected with coronaviruses. Morbidity can be close to 100%, and mortality, which is highest in young birds, varies from <10% to 50% or more (14,34). No vaccine currently exists for TCV. The gold standard for detecting TCV is virus isolation (VI) in specific-pathogen-free (SPF) turkey embryos followed by electron microscopy (EM). Unfortunately, large quantities of virus are necessary for visualization by EM. Currently, immunofluorescence assay (IFA) is used to screen flocks for TCV antibodies. This assay requires TCV-infected turkey embryo intestines as well as expensive equipment and highly trained personnel. Recently, a competitive enzyme-linked immunosorbent assay (cELISA) was described for detection of TCV antibodies (12). Once TCV is diagnosed, contaminated farms should be thoroughly disinfected (22). In addition, management practices should be put in place that will limit the risk of transporting the virus to additional facilities. Until recently (7), when TCV was detected among commercial turkey flocks in Europe, TCV had only been confirmed in the United States and Canada.

Astroviruses are small, positive-sense RNA viruses (27–30 nm) that cause enteric disease in several species, including humans (21). Little is understood about astrovirus infections of turkeys (10,18,23,24, 25,26,27,31,32), and detection has been dependent on VI and EM. No serologic assay has been reported for TAsVs. We recently isolated and characterized a novel astrovirus associated with PEMS-affected

turkey flocks (17,18,27). This turkey astrovirus isolate (TAsV-2) causes enteritis, lymphoid tissue atrophy, and mortality in young turkeys (15). With a reverse transcription–polymerase chain reaction (RT-PCR) test (17), the TAsV-2 was found to be circulating in diseased flocks in turkey-rearing regions of the United States and Canada.

Viral agents associated with enteritis are difficult or impossible to culture *in vitro*. Identification of these two viruses is dependent on VI followed by EM or serologic tests. VI and EM are costly and time consuming. While serologic detection of antibodies is convenient and inexpensive, seroconversion can take up to several weeks and can postpone diagnosis. Increasingly, PCR and RT-PCR assays are becoming incorporated into poultry diagnostic laboratories. However, the need to perform separate, individual assays for each virus increases the time and costs of the diagnostic test. The multiplex RT-PCR was recently described for the detection of several avian respiratory viruses (1,20,30). Multiplex RT-PCR can be incorporated into the diagnostic routine as an effective means of screening flocks for multiple organisms and contribute to effective management of outbreaks. The aim of these studies was to develop a multiplex RT-PCR test to identify TCV and TAsV-2 in commercial turkeys from the intestines/feces.

MATERIALS AND METHODS

Viruses. The TCV isolate originated from a commercial turkey flock in Texas and was isolated, purified, and propagated as described (11) with slight modifications. Briefly, embryo intestines containing TCV were homogenized in phosphate-buffered saline (PBS) in a dounce homogenizer and clarified by low-speed centrifugation. Supernatant was passed through a Whatman GD/X 0.2- μ m filter (Fisher Scientific, Norcross, GA), and filtrates were inoculated into the yolk sac of 23-day-old specific pathogen free (SPF) embryonated turkey eggs (NADC, Ames, IA) and incubated for 3 days at 37 C with humidity. After 3 days incubation, the small intestines were collected and stored at –80 C for viral or RNA isolation. TAsV-2 was isolated, purified, and propagated as described (17,18,27). Viral stocks were negatively stained and analyzed by electron microscopy at the College of Veterinary Medicine Electron Microscopy Laboratory, University of Georgia.

Field samples. Lower intestines and feces from commercial turkey flocks exhibiting enteritis, as well as from apparently normal flocks, were submitted to the laboratory and stored at –80 C until RNA was extracted.

RNA isolation and RT-PCR. Total RNA was isolated from either purified virus (18), embryo

intestines inoculated with a combination of TCV and TAsV-2, uninoculated embryo intestines, or directly from the intestines/feces of field samples submitted from commercial turkey flocks using Trizol™ (Invitrogen Life Technologies, Carlsbad, CA) following manufacturer's instructions. Initially (protocol one, see below) viral cDNA was obtained by incubating 1 µg of the extracted total RNA with 10 pmol of reverse primer(s) for TAsV-2 or 20 pmol of reverse primer for TCV or a combination of the primers on combined RNA (multiplex RT-PCR) in a 20 µl reaction containing 1.5 units of Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen Life Technologies) and 20 pmol of deoxynucleoside triphosphates (dNTPs) at 37 C for 60 min (17). In protocol two, 200 units of Superscript II reverse transcriptase were used in the first strand reaction at 42 C for 60 min. For both procedures, reverse transcriptase activity was inactivated at 70 C for 15 min.

Primers. The TAsV-2-specific primers MKPOL10 (forward) and MKPOL11 (reverse) (5'TGGCGGCGAACTCCTCAACA3' and 5'AATAAGGTCTGCACAGGTCG3', respectively) were developed previously and amplified an 802-bp fragment within the polymerase gene of TAsV-2 (17).

Although the TCV genome has not been completely sequenced, there is sequence information reported for the matrix (M) and nucleocapsid (N) genes (4,5). The avian coronavirus N gene sequences were aligned using Lasergene software (DNASTar Inc. Madison, WI) and used to design primers specific to conserved regions (Genbank Accession numbers: M28565, AF111995, AF111996, and AF111997). Coronavirus N primers TCVnucleo forward and TCVnucleo reverse (5'GGTAGCGGTGTTCTCTGA3' and 5'CCCTCCTTACCCTTAGT3', respectively), which amplify a 598-bp fragment within the N gene of TCV and IBV (data not shown), were selected for use in these assays. PCR products were purified by Qiaquick PCR purification system (Qiagen, Valencia, CA) and sequenced to confirm amplification of the intended gene (data not shown).

Multiplex RT-PCR. For multiplex RT-PCR, TCV, and TAsV-2 viral RNA were reverse transcribed simultaneously, and PCR was performed with the primer pairs for each virus.

Protocol 1. The PCR reaction contained an aliquot (2 µl) of the first strand product, 10 pmol of primers, 20 pmol of dNTPs, 1.5 mM MgCl₂, and 1.5 units of *Taq* DNA polymerase (Invitrogen Life Technologies) in a 50 µl reaction. Amplification, performed in a MJ Research DNA thermocycler (Waltham, MA), involved an initial denaturation step at 94 C for 5 min, followed by 35 cycles of 94 C for 45 sec, 53 C for 30 sec, and 72 C for 50 sec, with a final extension set at 72 C for 2 min. Ten microliters of PCR products were electrophoresed at 100 V for 1 hr in a 1.5% SeaKem LE agarose gel (FMC, Rockland, Maine) in 1 × Tris borate EDTA

(TBE) electrophoresis buffer and visualized by ethidium bromide staining and ultraviolet (UV) transillumination. Gel images were captured using the Kodak DC290 digital camera and Kodak 1D 3.5 digital documentation system software (Eastman Kodak, New Haven, CT). This protocol was used for PCR in all instances unless stated otherwise.

Protocol 2. Optimization of a multiplex RT-PCR requires compromises in concentrations of PCR reagents, annealing temperatures, and cycling conditions to attain the best strategy for amplification of more than one target sequence. The sensitivity of the multiplex assay was increased by making a few alterations to the above mentioned protocol one. Briefly, 5 µl of the first strand product was used as template for amplification in a 50 µl reaction containing 20 pmol of primers, 20 pmols of dNTPs, 3.0 mM MgCl₂, 2.5 units of *Taq* DNA polymerase (Invitrogen Life Technologies) and 1.6 × PCR buffer (supplied by Invitrogen Life Technologies). Amplification involved an initial denaturation step at 94 C for 5 min, followed by 35 cycles of 94 C for 45 sec, 53 C for 30 sec, and 65 C for 50 sec, with a final extension set at 65 C for 2 min. Ten microliters of PCR products were electrophoresed at 100 V for 1 hr in a 1.5% ultrapure agarose gel (BioRad, Hercules, CA) in 1 × Tris acetate EDTA (TAE) electrophoresis buffer and visualized by ethidium bromide staining and UV transillumination. Gel images were captured as described above.

Specificity and sensitivity of multiplex RT-PCR. To test the specificity of the multiplex RT-PCR assay with other common avian RNA viruses, amplification reactions were performed, as described above using 1 µg of RNA from reovirus (REO), infectious bursal disease virus (IBDV), Newcastle disease virus (NDV), and infectious bronchitis virus (IBV). The specificity of the assay was also evaluated similarly for common avian DNA viruses, adenovirus serotypes 1 and 8, chicken anemia virus (CAV), and infectious laryngotracheitis virus (ILT). To evaluate the detection limit of the multiplex RT-PCR test, total RNA from the intestines of embryos inoculated with TCV and TAsV-2 were assayed in half-log dilutions ranging from 1 µg to 1 ng, as determined by spectrophotometric analysis (BioPhotometer, Eppendorf, Hamburg, Germany), using viral-specific primers alone or combined primers (protocol 2) in the multiplex RT-PCR as described above. PCR products were electrophoresed at 100 V for 1 hr in a 1.5% ultrapure agarose gel (BioRad Labs, Hercules, CA) in 1 × TAE electrophoresis buffer and visualized by ethidium bromide staining and UV transillumination. Gel images were captured as described above.

RESULTS

Detection of TCV and TAsV-2 by multiplex RT-PCR. Initially, RNA was isolated from the

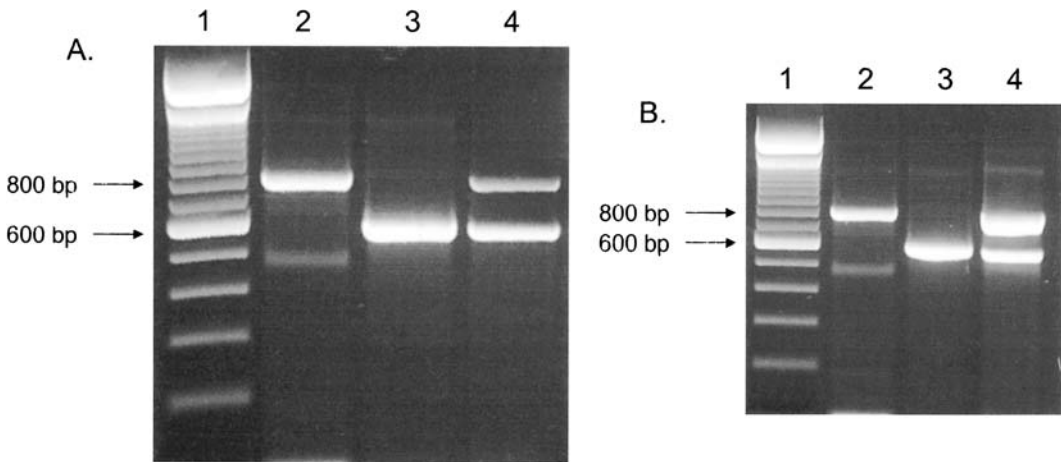


Fig. 1. Multiplex RT-PCR for TAsTV-2 and TCV. Total RNA was isolated from TAsTV-2-infected or TCV-infected embryos and RT-PCR performed using TAsTV-2 specific primers MKPOL10 and MKPOL11 amplifying an 802-bp product (lane 2A and 2B) or TCV-specific primers TCVnucleo forward and TCVnucleo reverse amplifying a 598-bp product (lane 3A and 3B). Multiplex RT-PCR was performed by combining 0.001 μ g TAsTV-2 RNA with 1 μ g TCV RNA (lane 4A) or coinfected embryos with TAsTV-2 and TCV and isolating 1 μ g total RNA (lane 4B) and performing RT-PCR with both sets of primers at the same time. The molecular weight ladder is a 100-bp ladder with the bright band representing 600 bp (lane 1A and 1B).

individual purified viruses and combined in a test tube, and RT-PCR was performed using a single set of primers or a combination of the primers that would detect both of the viruses (Fig. 1A). The viruses are easily differentiated by size (TAsTV-2 = 802 bp and TCV = 598 bp) in the multiplex RT-PCR. Based on these results, the viruses were combined and inoculated into SPF turkey embryos, and total RNA was isolated from the embryo intestines 5 days postinoculation and tested by single and multiplex RT-PCR (Fig. 1B). Similar to the results with the *in vitro* combined RNA, the multiplex RT-PCR assay detected both RNA species from a coinfected embryo tissue sample. Occasionally, after agar gel electrophoresis, a band artifact around 500 bp was present in the TAsTV-2 amplicon. This artifact was consistently absent in the multiplex amplicons.

Specificity and sensitivity of multiplex RT-PCR. The specificity of the multiplex RT-PCR primers was tested using RNA templates from REO, IBDV, NDV, and IBV and DNA templates from adenovirus serotypes 1 and 8, CAV, and ILTV. As expected, only IBV produced an RT-PCR product of 598 bp. No amplicons were observed for the other viruses (data not shown).

The detection limit of individual RT-PCRs for TCV (Fig. 2A) and TAsTV-2 (Fig. 2B) was

determined to be between 5 and 1 ng (lanes 7 and 8, respectively). Similarly, with protocol 2 (described above) the detection limit in the multiplex RT-PCR was determined to be between 10 and 5 ng (Fig. 2C, lanes 6 and 7, respectively). Positive bands were observed for TAsTV-2 in the multiplex below the 5-ng limit, while a faint band was observed for TCV at the 5-ng limit.

Detection of TCV and TAsTV-2 from field samples. Pooled lower intestines/feces from commercial turkey flocks in Texas (Fig. 3A lane 3), North Carolina (Fig. 3A, lanes 4, 5, and 7), Missouri (Fig. 3A, lanes 6 and 8), and Minnesota (Fig. 3A, lane 11) were inoculated into embryonated turkey eggs and tested for TCV and TAsTV-2. The multiplex RT-PCR assay amplified both TCV and TAsTV-2 in the embryo passaged field samples (Fig. 3A).

To determine the utility of this assay directly on field material, RNA was extracted directly from intestines/fecal samples from commercial turkeys exhibiting enteritis and growth depression. Samples 31498, 31489, and 31485 (Fig. 3B lanes 2, 4, 6, respectively) were positive for both TCV and TAsTV-2 nucleic acids. Sample 31479 was positive for TAsTV-2 and 31308 was positive for TCV (Fig. 3B lanes 3 and 5, respectively). Both TCV and TAsTV-2 were recognized in turkey intestines without prior passage in turkey embryos.

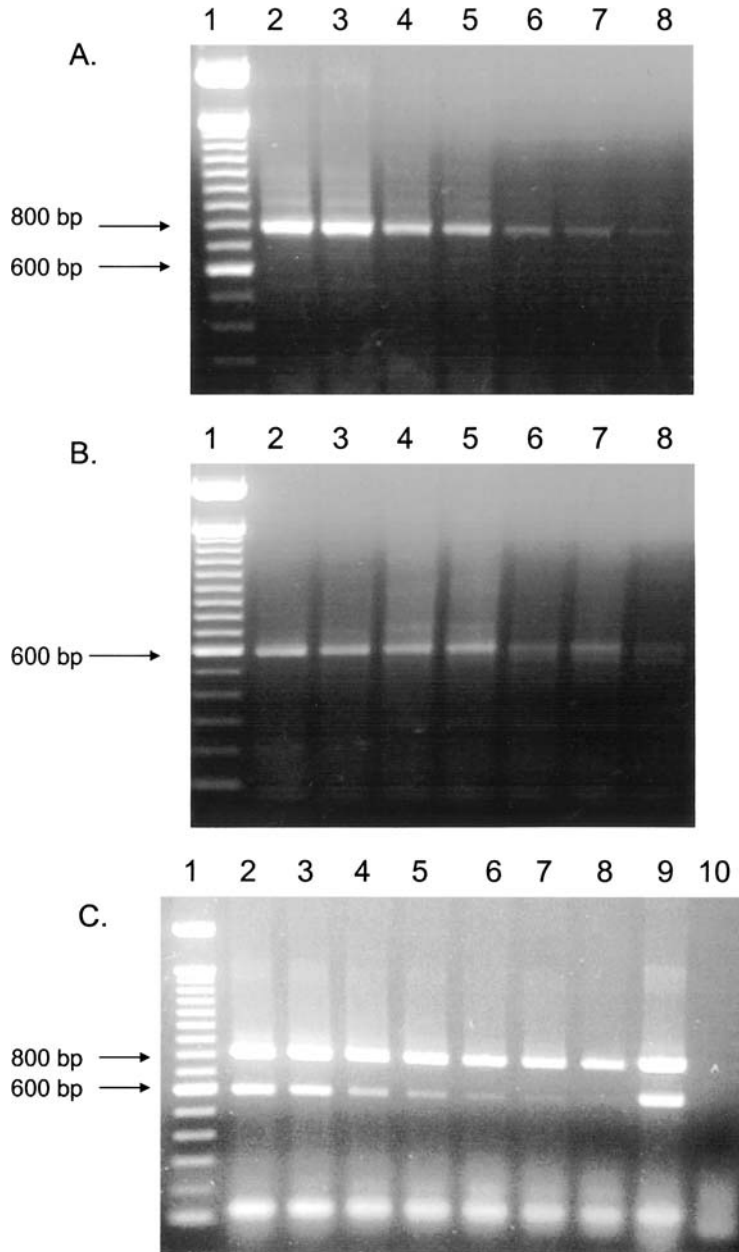


Fig. 2. Sensitivity of the multiplex RT-PCR. (A) RT-PCR performed using TAsV-2 specific primers MkpPOL10 and MkpPOL11 amplifying a 802-bp product, (B) TCV-specific primers TCVnucleo forward and TCVnucleo reverse amplifying a 598-bp product; and (C) multiplex RT-PCR using MkpPOL10, MkpPOL11, TCVnucleo forward and reverse primers. Total RNA was isolated from turkey embryos coinfecting with TAsV-2 and TCV, and RT was performed using 1 μ g total RNA (lane 2A, 2B, and 2C), 0.5 μ g total RNA (lane 3A, 3B, and 3C), 0.1 μ g total RNA (lane 4A, 4B, and 4C), or 0.05 μ g total RNA (lane 5A, 5B, and 5C), 10 ng total RNA (lane 6A, 6B, and 6C), 5 ng total RNA (lane 7A, 7B, and 7C), and 1 ng total RNA (lane 8A, 8B, and 8C). The molecular weight ladder is a 100-bp ladder with the bright band representing 600 bp (lane 1A, 1B, and 1C). Lanes 9 and 10 contain the positive TCV/TAsV-2 multiplex control and negative multiplex RT-PCR control, respectively.

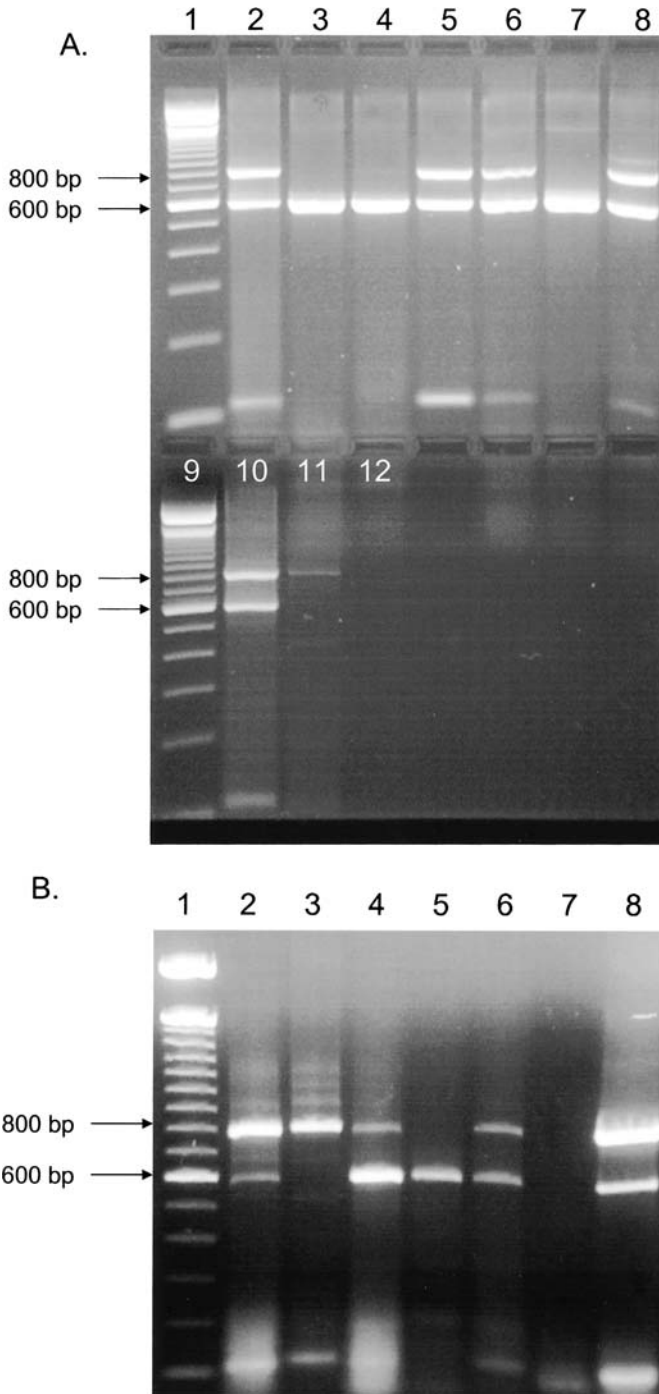


Fig. 3. Multiplex RT-PCR using field isolates. (A) Total RNA was isolated from turkey embryos inoculated with an intestinal/fecal tissue filtrate collected from commercial turkey flocks exhibiting enteritis and tested by multiplex RT-PCR. Lanes 1 and 9 = 100-bp molecular weight with the bright band representing 600 bp; lanes 2 and 10 = embryo intestines coinfecting with TAsV-2 and TCV (positive multiplex RT-PCR controls); lane 3 = a turkey flock in Texas; lane 4 = North Carolina, company 1; lane 5 = North Carolina, company 2; lane 6 =

DISCUSSION

In this study, we developed a rapid multiplex RT-PCR to simultaneously detect nucleic acid from two important enteric pathogens of turkeys, TCV and TAsV-2. The multiplex RT-PCR was designed to detect nucleic acid directly from intestines/feces of turkeys without passage in SPF turkey embryos. Products of the expected size, 598 bp, were amplified from TCV, as well as IBV, and an 802-bp product was amplified from TAsV-2. The multiplex PCR did not amplify RNA or DNA from other common avian viruses. A close genomic and antigenic relationship has been established for IBV and TCV (6,7,11). Genomic analyses of the N gene of TCV and IBV reveals a 90% similarity (6). Detection of both TCV and IBV using the TCV nucleo forward and reverse primers was expected, since the primers were designed to conserved regions of the N gene of both viruses. There does not appear to be a need to discriminate between TCV and IBV, since both viruses have a limited host range. Chickens and pheasants are the only known natural hosts for IBV (9,29). Experimental infection of turkeys with IBV and chickens with TCV has not been successful (Mark Jackwood, pers. comm.). Therefore, in this diagnostic assay the goal was to detect any coronavirus from intestines/fecal material from turkeys.

The detection limit of the TCV and TAsV-2 primers in the multiplex PCR was approximately 10 ng using protocol 2. The amplification product obtained for 10 ng of RNA template was clearly visible in a 1.5% agarose and ethidium bromide stained gel, whereas 5 ng of template produced a faint band still visible in the stained gel. The detection limit was not compromised in the

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Missouri; lane 7 = North Carolina, company 3; lane 8 = Missouri, company 2; lane 11 = Minnesota; lane 12 = negative multiplex RT-PCR control. The TAsV-2 primers amplify an 802-bp amplicon, and the TCV primers amplify a 598-bp amplicon. (B) Total RNA was isolated directly from intestine/fecal tissue from commercial turkeys exhibiting enteritis and tested by multiplex RT-PCR. Lane 1 = 100 bp molecular weight marker with the bright band representing 600 bp; lanes 2, 4, 6 = positive TCV and TAsV-2 field samples 31498, 31489, and 31485; lane 3 = positive TAsV-2 field sample 31479; lane 5 = TCV positive field sample 31308; lane 7 = negative multiplex RT-PCR control; lane 8 = Positive TCV/TAsV-2 multiplex RT-PCR control.

multiplex format as seen by comparison of detection limits obtained from individual TCV and TAsV-2 RT-PCRs. The detection limit of the multiplex assay was determined using embryo-passaged intestinal material and may be decreased as a result of potential inhibitory agents present in clinical samples. Our test was not quantitative. Thus, we are unable to determine the concentration or ratio of TCV to TAsV-2 in the embryo intestines.

Evidence of simultaneous TCV and TAsV-2 infection was observed in field samples from across the United States. In most cases, these results could be obtained weeks in advance of virus isolation and seroconversion. Given that there is no vaccine for either virus, control of the diseases is extremely difficult, especially in multiage farms, which are common in the turkey industry. Detection of TCV and TAsV-2 in the early stages of the disease is imperative so that strict containment and biosecurity principles can be implemented.

The multiplex RT-PCR has several advantages over VI and serologic tests, specifically, early detection and rapid screening of flocks. The results of the multiplex RT-PCR can be obtained in less than a day, whereas virus isolation followed by EM can take up to several weeks to complete. While serologic detection by ELISA is also a means for screening flocks, seroconversion can take up to several weeks, thus postponing an important diagnosis.

In summary, the TCV and TAsV-2 multiplex RT-PCR assay is currently being used to detect two economically important enteric viruses in commercial turkey flocks. The multiplex test can be completed rapidly and provides significant savings in time, materials, and cost to the company, as compared to individual RT-PCR tests, indirect immunofluorescent assays, or ELISA.

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