A reverse transcriptase–polymerase chain reaction assay for the diagnosis of turkey coronavirus infection

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Abstract.   This study reports on the development of a reverse transcriptase–polymerase chain reaction (RT-PCR) for the specific detection of turkey coronavirus (TCoV). Of the several sets of primers tested, 1 set of primers derived from the P gene and 2 sets derived from the N gene of TCoV could amplify the TCoV genome in the infected samples. The RT-PCR was sensitive and specific for TCoV and did not amplify other avian RNA and DNA viruses tested except the infectious bronchitis virus (IBV). To overcome the problem of IBV amplification, a set of separate primers was designed from the spike protein gene of IBV. The RT-PCR under the same conditions as above could effectively differentiate between TCoV and IBV. The closely related bovine coronavirus and transmissible gastroenteritis virus of pigs were differentiated from TCoV using the same RT-PCR with slight modifications. The results of RT-PCR correlated well with the results of the immunofluorescent test for the same samples tested at the Purdue University Animal Disease Laboratory, West Lafayette, Indiana. The nucleotide sequence and projected amino acid sequence comparison of the P gene of different isolates of TCoV from 5 different states in the United States revealed a close association among the different isolates of TCoV.

Coronavirus belongs to the family Coronaviridae, a large group of ribonucleic acid (RNA) viruses that infect a wide range of avian and mammalian species. They are pleomorphic positive-sense single-stranded RNA viruses measuring 60–220 nm in diameter.[3,11] The characteristic feature of the coronaviral morphology is the presence of long (12–24 nm), widely spaced petal- or pear-shaped surface projections, which impart to the virus the appearance of a solar corona.[14]

Three structural proteins have been identified in the virus. They are the spike or peplomer (S) protein (90–180 kD), membrane (M) protein (20–35 kD), and the nucleocapsid (N) protein (50–60 kD). A fourth type of protein called the hemagglutinin-esterase (120–140 kD) is seen in some groups of viruses.[14]

The members of this family are classified into 3 groups on the basis of immunofluorescent assay (IFA), enzyme-linked immunosorbent assay (ELISA), immunoelectron microscopy (IEM), and nucleotide sequence analysis. Group 1 consists of human coronavirus 229E, transmissible gastroenteritis virus (TGEV), canine coronavirus, and feline infectious peritonitis virus. Group II consists of human coronavirus OC43, murine hepatitis virus, and bovine coronavirus (BCV). Infectious bronchitis virus (IBV) and turkey coronavirus (TCoV) belong to group III of Coronaviridae.[3,7,15]

Turkey coronavirus causes an acute, highly infectious disease affecting turkeys of all ages. The disease is known by various names. Blue comb disease, mud fever, transmissible enteritis, and infectious enteritis are synonymously used to describe coronaviral enteritis.[8,12] This disease is of high economic importance because it causes poor production performance, impaired growth, poor feed conversion, and mortality. Coronaviral enteritis was the most costly disease of turkeys in Minnesota between 1951–1971. It was eradicated from Minnesota by 1976. The disease reappeared in Indiana in 1996, and it occurred in North Carolina in 1997. The clinical signs of this infection include inappetence, wet droppings, ruffled feathers, weight loss, depression, and uneven flock growth. The morbidity is high, and the mortality varies.

The common diagnostic tests for coronaviral enteritis of turkeys include virus isolation, virus neutralization, direct/indirect FA,[13] IEM, immunoperoxidase test, and ELISA.[5,11] The most widely used test for the serologic diagnosis of coronaviral enteritis is the indirect fluorescent antibody test. These procedures are labor intensive and time consuming. In addition, they require expensive equipment, highly trained personnel, and an antigen obtained from the frozen sections of TCoV-infected turkey embryo intestines or epithelial cells exfoliated from the Bursa of Fabricius or from infected kidneys. As far as TCoV-specific ELISA is concerned, the practical difficulty in propagating the virus on cell cultures makes it an undesirable procedure because a large amount of high-quality antigen is required for this purpose.

As a diagnostic tool, reverse transcriptase–polymerase chain reaction (RT-PCR) is a highly specific, sen-

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**Table 1.** Primers used for the amplification of TCoV genome.

| Primer designation	Primer sequence	Target gene	Location	Expected size of the PCR product (bp)
N1-upper	5′-CAGCGCCAGTCATCAAAC	N	38	
N2-lower	5′-TGGTCAAACTTGTCAGGGTCC	N	417	380
N3-upper	5′-CAAGTAAAGGCGGAAGAAAAC	N	235	
N4-lower	5′-GCCTTAGTAATGCGAGAGCCC	N	651	417
P1-upper	5′-CAGTGGCAGGTGTGTCTATCC	P	150	
P2-lower	5′-GACTCGCCTGTAAACCTGCTGG	P	688	539

sitive, efficient, and rapid test to screen large numbers of samples. It can detect the genome even in trace amounts. Consequently, the objectives of the present study were to develop an RT-PCR for detection of TCoV, to test the sensitivity and specificity of the assay, to evaluate the assay using known positive and negative samples, and to do the sequence comparison of different isolates of TCoV.

The Minnesota isolate of TCoV (TCoV/Minnesota N1) was used for the standardization of the assay. Turkey eggs obtained from a breeder flock in Minnesota, known to be free of TCoV infection, were used to propagate the virus. They were inoculated with TCoV at 24 days of incubation by amniotic cavity. Embryos were harvested after 3 days of incubation at 37 C, and the intestines of turkey embryos were homogenized in minimum essential medium (MEM)[a] with penicillin (0.5 U/ml) and streptomycin (0.5 μg/ml). The homogenate (20% w/v) was centrifuged at 8,000 × g for 10 minutes, and the supernatant was used. Viral RNA was prepared from the supernatant of intestinal homogenate using a viral RNA extraction kit[b] according to the manufacturer's protocol. The viral RNA was first reverse transcribed into complementary DNA (cDNA) and then amplified using an RT-PCR kit.[c] The primers were designed on the basis of the published cDNA sequences of N and P genes (GenBank accession numbers AF 111996 and AF 124991, respectively) of TCoV. One set of forward and reverse primers was designed from the P gene, and 2 sets were designed from the N gene of TCoV (Table 1). The conditions for amplification included reverse transcription at 50 C for 30 minutes, an initial denaturation at 94 C for 15 minutes followed by repeated 35 cycles of annealing at 55 C for 1 minute, extension at 72 C for 1 minute and denaturation at 94 C for 30 seconds, followed by a final extension at 72 C for 10 minutes. A known IFA-positive TCoV isolate from North Carolina was used as the positive control with primers P1–P2, and an uninfected turkey embryo intestinal homogenate was used as the negative control. The products of RT-PCR were then analyzed on 1.2% agarose gel. The identity of PCR products was confirmed by DNA sequencing. The 3 sets of primers designed, 1 from the P gene and 2 sets from the N gene, amplified the TCoV genome (Fig. 1).

To test the sensitivity of the assay, the supernatant of the intestinal homogenate (20% w/v) was further diluted in MEM into 10-fold dilutions. Viral RNA was extracted from each dilution using a viral RNA extraction kit,[b] and the amount of RNA in each dilution was measured using a spectrophotometer. The different dilutions of the sample were subjected to RT-PCR to find out the highest dilution of the sample showing
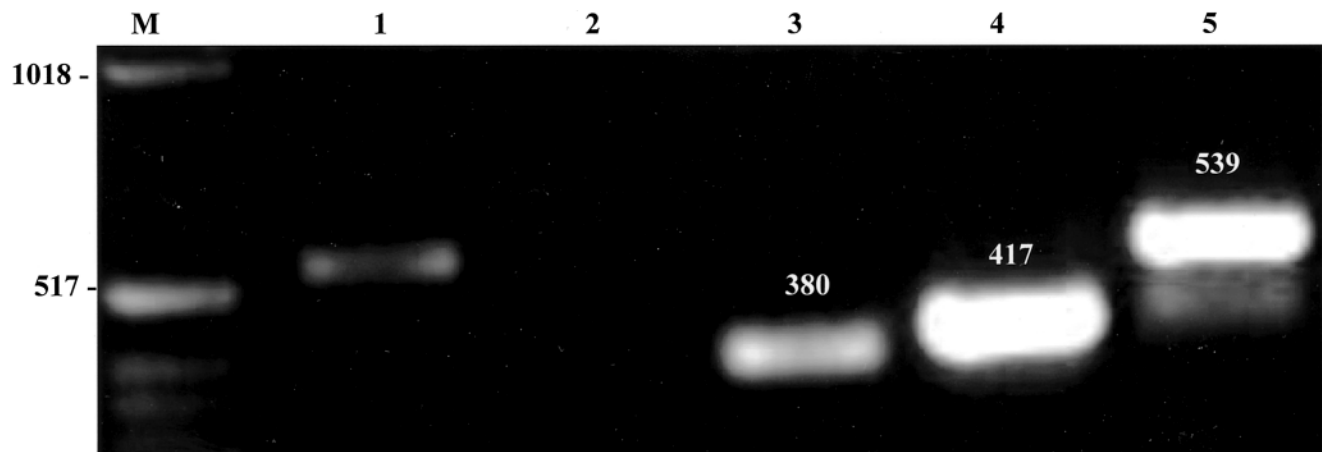
Figure 1. Reverse transcriptase–polymerase chain reaction (RT-PCR) of TCoV with different sets of primers. M, marker; 1, TCoV control; 2, negative control; 3, TCoV with primers N1–N2; 4, TCoV (N3–N4); 5, TCoV (P1–P2).
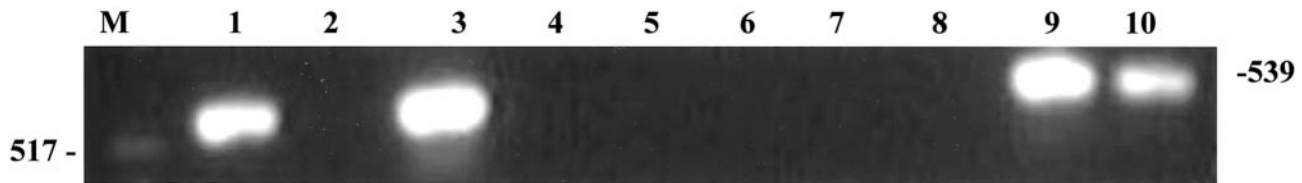
Figure 2. Specificity of RT-PCR to detect TCoV. The viral RNA extracted from TCoV, Newcastle Disease Virus (NDV), Infectious bursal disease virus (IBDV), Avian influenza virus (AIV), reovirus and IBV were tested by RT-PCR. M, marker; 1, PCR control; 2, negative control; 3, TCoV with primers P1–P2; 4, NDV; 5, IBDV virulent strain; 6, IBDV vaccine strain; 7, AIV; 8, reovirus; 9, IBV Arkansas type; 10, IBV mass type along with NDV.

positive results. The sensitivities of the reactions with the 3 different primer sets were as follows. Primers P1–P2: 10^{-4} dilution with a viral RNA concentration at the level of 10 ng/ml; primers N1–N2: 10^{-5} dilution with a viral RNA concentration of 1 ng/ml; and primers N3–N4: 10^{-4} dilution with a viral RNA concentration of 10 ng/ml (data not shown).

The specificity of these primers in detecting the TCoV genome was tested using other avian RNA and DNA viruses that included: Newcastle disease virus,[d] infectious bursal disease virus,[e] nonpathogenic avian influenza virus (University of Minnesota, Saint Paul, MN), reovirus (University of Minnesota), infectious laryngotracheitis virus,[f] avian encephalomyelitis virus,[g] several serotypes of IBVs (kindly provided by Dr. Syed Naqi, Cornell University, Ithaca, NY), poxvirus,[g] Mareks disease virus serotype 3 (University of Minnesota), and hemorrhagic enteritis virus of turkey (University of Minnesota). No amplification of DNA was observed in other avian RNA and DNA viruses except the IBV (Figs. 2, 3). All 3 sets of primers amplified the IBV genome as well.

To circumvent the problem of IBV amplification, primers were designed from the known sequence of the spike protein gene (S1, GenBank accession number AF 027511) of IBV (Sp1 forward primer, 5′-GTCTACTACTACCAAAGCGCC-3′ starting from base 79 and Sp2 reverse primer, 5′-AGTTA-TAGGTCCGCCGG-3′ starting from base 603 with an expected size of 525 bp), and the same conditions mentioned above were used for the RT-PCR. Primers designed on the basis of the spike protein genome of IBV amplified only IBV and not TCoV under the same conditions as with TCoV primers (Fig. 4).

The specificity of the RT-PCR was also checked by using BCV and TGEV of pigs. Bovine coronavirus (Mebus strain, Cell culture–adapted virus, BL1, BT14) and TGEV of pigs (Purdue-attenuated strain, P115G) used in this study were kindly provided by Dr. Linda Saif, Ohio State University, Columbus, Ohio. When BCV and TGEV of pigs were tested by this RT-PCR, the primer pair P1–P2 amplified only TCoV and not BCV and TGEV when the annealing temperature was increased to 60 C (data not shown).

The reliability of the RT-PCR assay was evaluated using known positive and negative samples of TCoV. A total of 16 positive and 12 negative TCoV samples were provided by the Purdue University Animal Disease Laboratory, West Lafayette, Indiana. They were tested using IFA. The RT-PCR reconfirmed the positive and negative samples tested by IFA earlier. The results of RT-PCR correlated very well with the results of the IFA (data not shown). These samples were also cross-checked with the IBV spike protein gene-derived primers. None of the samples were positive (data not shown), confirming that they were not infected with IBV.

The P gene segment of 539 bp length starting from base 150 to base 688 of 5 isolates of TCoV obtained from Minnesota, North Carolina, Missouri, Arkansas, and Virginia was sequenced. The nucleotide sequence similarity and projected amino acid identity were compared. Comparison of the nucleotide sequence of the 539-bp-long segment of the P gene from the different isolates showed 82–93.4% sequence similarity among the 5 different isolates (data not shown). The predicted amino acid sequence comparison revealed a close association (about 90%) among the 5 different isolates.
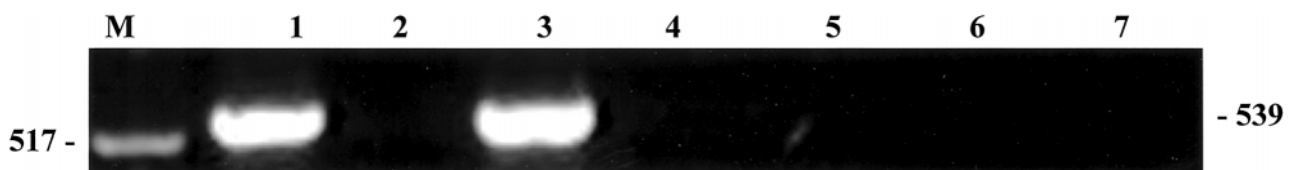
Figure 3. Reverse transcriptase–polymerase chain reaction (RT-PCR) of TCoV with Hemorrhagic enteritis virus of turkey (HEVT), Infectious laryngotracheitis virus (ILTV), Mareks Disease virus (MDV), Avian encephalomyelitis virus (AEMV) and poxvirus. M, marker; 1, PCR control; 2, negative control; 3, TCoV with primers P1–P2; 4, HEVT; 5, ILTV; 6, MDV; 7, AEMV along with poxvirus.
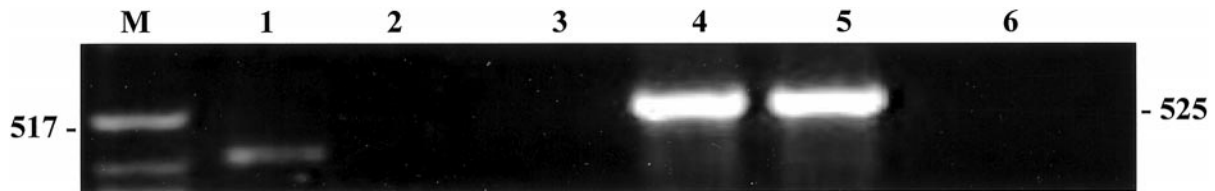
Figure 4. Reverse transcriptase–polymerase chain reaction (RT-PCR) of TCoV and IBV with primer derived from the spike protein gene of IBV. M, marker; 1, PCR control; 2, negative control; 3, TCoV with primers Spl–Sp2; 4. IBV Arkansas type with primers Sp1–Sp2; 5, IBV mass type along with NDV with primers Sp1–Sp2; 6, NDV with primers Sp1–Sp2.

In the present study, an RT-PCR was developed for the specific detection of TCoV in the intestinal samples of affected birds. Of the various diagnostic tests available, virus isolation (VI), fluorescent antibody (FA), and immunoperoxidase test (IP) are highly specific but they are time consuming. RT-PCR has been shown to be highly sensitive, specific, and rapid compared with conventional diagnostic procedures.[2]

The N protein of coronavirus binds to the virion RNA and provides the structural basis for the helical nucleocapsid.[10] It is the most abundant viral polypeptide in coronavirus-infected cells, and it is an immunologically dominant polypeptide.[8,10] The most highly conserved genomic sequences are found in the 20-kb polymerase gene, which covers the 5′ two thirds of the coronavirus genome. An evolutionary link among coronaviruses, arteriviruses, and toroviruses has been demonstrated on the basis of these conserved functions and similarities in replication and expression strategies.[4,15] This makes the viral polymerase gene a logical region for making phylogenetic comparisons[15] as well as a potential candidate for PCR assay. The P gene was selected as one of the genes for this RT-PCR because of the conserved nature of the gene. This could also lead to the identification of novel coronaviruses.[15]

The RT-PCR could specifically amplify the TCoV genome, and no DNA amplification was observed with other avian RNA and DNA viruses except IBV. Recent studies have strongly indicated a close antigenic as well as genetic relationship between these 2 important viruses.[1,3,7,15] The complete nucleotide sequence of the N gene and a portion of the M gene were analyzed, and their projected amino acid sequences were found to be very similar (more than 90%) to those of IBV M and N proteins.[1,3,15] The amplification of the IBV genome can be explained on the basis of the above-mentioned observation.

The intestinal samples from turkey poults received from the Purdue University Animal Disease Laboratory consisted of 16 positive and 12 negative samples tested by IFA. The RT-PCR presented here confirmed the findings of IFA. Some researchers have pointed out that the intestinal contents might inhibit the enzymatic reactions of RT-PCR giving false-negative results.[9,18] Extensive processing of intestinal contents was rec-

ommended to eliminate the false-positive results and for the successful application of the RT-PCR procedure for TCoV detection.[2] But it was demonstrated that the supernatant of the intestinal homogenate after centrifugation could very well be used for the detection of TCoV. There were no false-negative results when the 16 positive samples tested by IFA were retested by RT-PCR. This makes the procedure simple and highly efficient, especially when large numbers of samples are to be screened.

When the same RT-PCR was used to test BCV and TGEV, which belong to the same family, Coronaviridae, there were amplification products at 55 C but not at 60 C of annealing temperature. This can be attributed to the nonspecific amplification at lower annealing temperatures. Another reason can be the close genomic relationship between TCoV and BCV, which was established by sequence analysis and hybridization.[6,16,17]

The nucleotide sequence comparison of the P gene of 5 different isolates of TCoV revealed 88–96% similarity among the isolates. The P gene is found to be the most highly conserved gene of coronaviruses.[15] The nucleotide sequence similarity and projected amino acid sequence identity based on the P gene suggests that the 5 different isolates of TCoV are very closely related. Although these conclusions are made by comparing a sequence of 539 bp of P gene, one cannot conclusively establish similarity of these isolates without analyzing the complete genome.

The present study was aimed at the development of RT-PCR for the specific and rapid diagnosis of TCoV. The assay could efficiently detect the TCoV genome in intestinal samples of affected birds. The hurdles caused by the amplification of the IBV genome could be overcome by designing another set of primers based on the spike protein gene of IBV. The nucleotide and deduced amino acid sequence comparison suggested a close relationship among the different isolates of TCoV. This can possibly unfold the development of an effective vaccination program based on the conserved gene sequences. Turkey coronavirus is still an important disease entity in turkey flocks. Early and specific detection of the disease will facilitate economic farming and control of the disease.

Sources and manufacturers

a. Minimum essential medium, GIBCO BRL, Chagrin Falls, OH.
b. QIAamp viral RNA mini kit, Qiagen, Valencia, CA.
c. One-step RT-PCR kit, Qiagen, Valencia, CA
d. B$_1$ type, Lasota strain, University of Minnesota, Saint Paul, MN.
e. Virulent strain IM and vaccine strain B$_2$, University of Minnesota, Saint Paul, MN.
f. Fowl Laryngotracheitis vaccine, modified live virus, Fort Dodge Animal Health, Fort Dodge, IA.
g. AE-Poxine, Live virus, Fort Dodge Animal Health, Fort Dodge, IA.

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