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Development of a Polymerase Chain Reaction Procedure for Detection of Chicken and Turkey Parvoviruses

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SUMMARY. Comparative sequence analysis of six independent chicken and turkey parvovirus nonstructural (NS) genes revealed specific genomic regions with 100% nucleotide sequence identity. A polymerase chain reaction (PCR) assay with primers targeting these conserved genome sequences proved to be highly specific and sensitive to detecting parvoviruses in experimentally infected chickens. In a nationwide survey, a total of 138 field enteric samples from poultry flocks were tested by PCR for parvovirus presence. Of the tested chicken samples that were collected in 54 farms, 77% showed the presence of parvovirus, while 78% of the turkey samples that were received from 29 farms were parvovirus positive. For the first time, our data clearly demonstrate that parvoviruses are widely distributed in commercial poultry flocks in the United States. The high prevalence of parvovirus infection in birds from enteric disease-affected flocks suggests a potential role of these viruses in the etiology of enteric disease of poultry. Phylogenetic analyses comparing NS gene segments showed that most of the chicken and turkey parvovirus isolates formed separate phylogenetic groups. These findings suggest that the chicken and turkey parvoviruses might have diverged from a common ancestor and have subsequently undergone host-specific adaptation.

RESUMEN. Desarrollo de un procedimiento de reacción en cadena por la polimerasa para la detección de parvovirus de pollo y pavo.

Mediante el análisis comparativo de los genes no estructurales de seis parvovirus de pollo y pavo independientes, se reveló la existencia de regiones genómicas específicas que compartían una identidad del 100% en sus secuencias. Mediante la utilización de iniciadores dirigidos para estas regiones conservadas, se demostró que un procedimiento de reacción en cadena por la polimerasa (de las siglas en inglés PCR) era altamente específico y sensible para detectar parvovirus en pollos infectados experimentalmente. En un muestreo realizado en los Estados Unidos con el fin de detectar la presencia del parvovirus, se analizaron mediante la PCR un total de 138 muestras de intestinos recolectadas en parvadas avícolas en el campo. De las muestras de pollo recolectadas de 54 granjas y que fueron analizadas, el 77% mostró la presencia del parvovirus, mientras que de las muestras recibidas de 29 granjas de pavos, el 78% de las muestras fueron positivas a la presencia de dicho virus. Nuestros datos muestran por primera vez, que los parvovirus están ampliamente distribuidos en las parvadas avícolas comerciales de los Estados Unidos. La alta prevalencia de la infección de parvovirus en aves con problemas entéricos sugiere el papel potencial de estos virus dentro de la etiología de enfermedades entéricas de las aves comerciales. El análisis filogenético que comparó los genes no estructurales mostró que la mayoría de los aislamientos de parvovirus de pollo y pavo se distribuyeron en grupos filogenéticos separados. Estas observaciones sugieren que los parvovirus de pollo y pavo pudieron haber evolucionado a partir de un ancestro común y posteriormente, desarrollaron una adaptación específica para cada huésped.

Key words: parvovirus, PCR, chicken, turkey, poultry, enteric disease

Abbreviations: bp = base pair; CEF = chicken embryo fibroblast; CEL = chicken embryo liver; DPI = days postinfection; ITR = inverted terminal repeat; LB = Luria Bertani; nm = nanometer; NS = nonstructural; nt = nucleotide; PEC = poult enteritis complex; PEMS = poult enteritis mortality syndrome; PCR = polymerase chain reaction; RSS = runting–stunting syndrome; SEPRL = Southeast Poultry Research Laboratory; SPF = specific-pathogen-free; VP = viral protein

Enteric diseases of poultry cause significant economic losses because of decreased bird weight gain, increased morbidity and mortality, and increased production costs from both poor feed conversions and the cost of treatment. Pathogens involved in enteric disease include bacteria, protozoa, and viruses. Currently, the role of these pathogens in the etiology of enteric diseases of poultry is not completely understood.

Two major viral enteric diseases occur in poultry: poult enteritis complex (PEC) in turkeys and runting–stunting syndrome (RSS) in chickens (2,12,20,21,24,26,31). Poult enteritis mortality syndrome (PEMS) of turkeys is a more-severe disease that is characterized by higher mortality rates (1). The etiologic agents of poultry enteric diseases are likely many and include potential candidates such as reovirus, coronavirus, rotavirus, and astrovirus (9,10,14,19,21,22, 23,24,25,27,30,31,37). Such viruses are sometimes isolated from birds both with and without disease, suggesting that pathotypic variation occurs between the viruses (21,24) or that a certain combination of pathogens or factors is required for disease presentation. A vaccine for PEC, PEMS, or RSS has not yet been developed because of the ambiguity concerning the exact etiologic agents of these diseases. Management of poultry enteric diseases includes cleaning and disinfection of poultry houses, increased biosecurity, and antimicrobial therapy to reduce the affect of bacterial coinfection (1,2).

Parvoviruses have also been identified in turkeys and chickens exhibiting enteric disease characterized by stunting, diarrhea, and mortality (18,36,39). Inoculation of day-old broilers with purified parvovirus particles resulted in characteristic clinical signs of RSS (16). Although parvoviruses are common pathogens in other species, the prevalence of parvoviruses in chicken and turkey flocks has not been studied using more-definitive methods, and the role of these viruses in the etiology of enteric disease in poultry has not been further investigated.

Similar to many other parvoviruses, isolation of chicken and turkey parvoviruses in tissue cultures or embryonating eggs has, so

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far, been unsuccessful (39). Parvoviruses contain a small, linear single-stranded DNA molecule, between 4 and 6 kilobase pairs in length, encapsidated within a nonenveloped, icosahedral virion approximately 20 nm in diameter (34). Parvoviruses encode two major genes: a nonstructural gene on the left half of the coding sequence that encodes a small number of replication proteins; and a structural viral protein (VP) gene that occupies the right half (6). The genome sequence of the nonstructural (NS) gene appears to be highly conserved within the parvoviruses, and it is often used as a target for diagnostic polymerase chain reaction (PCR) (4,5,7,29).

Previously, we described a molecular screening method for the detection of novel viruses in intestinal samples of chickens and turkeys exhibiting characteristic signs of enteric disease (39). The technique is based on random amplification of particle-associated nucleic acids in clinical samples. Using this method, we successfully identified parvovirus DNA sequences in intestinal homogenates from affected birds. Sequence analysis of the left end of the genome, including the complete NS gene, demonstrated that the chicken and turkey parvoviruses were closely related to each other and representative of a novel member of the *Parvoviridae* family (39). Although our previous studies showed a high level of homology between chicken and turkey parvovirus NS genes (39), certain nucleotide polymorphisms have also been observed within those sequences (L. Zsak, unpubl. data).

Here, we report the development and application of a conventional PCR assay to detect parvoviruses in poultry samples. Comparative sequence analysis of parvovirus isolates from turkeys and chickens was performed to identify a primer set within the parvovirus NS gene for use in a PCR assay. Sensitivity and specificity of the PCR was tested with samples from experimentally infected chickens. The PCR assay was used in a survey of birds to determine the prevalence of parvovirus infection in commercial poultry flocks in the United States. Phylogenetic analysis of viruses that were detected in this study was also performed to further study the relationship between chicken and turkey parvoviruses.

MATERIALS AND METHODS

Virus. The chicken parvovirus ABU strain was originally isolated from chickens in Hungary in 1984 (18). For further studies, the ABU virus was subsequently purified by cesium chloride density-gradient centrifugation (16). The cesium chloride-purified ABU virus (kindly provided by J. Kisary) was used to prepare virus as inoculum for chickens, as previously described (16). Briefly, 1-day-old specific-pathogen-free (SPF) white-rock broiler chickens ($n = 10$) were received from the Southeast Poultry Research Laboratory (SEPRL) flocks and inoculated orally with the cesium chloride-purified ABU virus. Seven days later, the chickens were killed and the entire intestinal portion was collected for further processing. A 10% homogenate of intestinal tissue was prepared in phosphate-buffered saline and clarified by centrifugation at 775 × *g*, and the supernatant was used to inoculate the chickens.

Chickens. SPF white-rock chickens were obtained from the SEPRL flocks at 3 days of age and divided into two groups of 15 birds each. Among other agents, these flocks are monitored for avian reovirus, adenovirus, infectious bursal disease virus, and chicken anemia virus (31). Chickens were housed in Horsfal isolators (Federal Designs, Inc., Comer, GA) with *ad libitum* access to feed and water. General care was provided as required by the Institutional Animal Care and Use Committee, as outlined in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (8).

Experimental infection of chickens with ABU parvovirus. One group of 15 birds (group A) was inoculated at 3 days of age with 0.1 ml of the chicken parvovirus ABU strain by oral routes. The other group (group B) was not inoculated and was kept as control birds. Cloacal swabs and plasma were collected at 4, 7, 10, 14, 21, 28, and 35 days postinfection (DPI) from each bird and were processed for DNA extraction using DNeasy blood and tissue kit (Qiagen, Inc., Valencia, CA), including 5 μg of carrier RNA. These DNA samples then were used in PCR amplification with a parvovirus-specific PVF1 and PVR1 primer set (see details for PCR assay below), and a total of 20 randomly selected positive amplicons were cloned and sequenced.

Clinical samples. Enteric samples were collected from commercial turkey and chicken flocks from different regions of the United States. The field samples were received as whole guts or fecal-composite samples. A total of 138 chicken and turkey samples consisted of pooled intestines, from three birds per house, from each of a total 54 chicken and 29 turkey farms. Sixty samples were from chicken or turkey flocks less than 2 wk of age; 78 samples were collected from birds between 2 and 7 wk of age. Information on flock condition or performance was not provided for all samples. Samples were collected between 2003 and 2008. All samples were preserved at 4 C or −70 C until shipment on wet-ice (by overnight courier) to SEPRL (USDA, Athens, GA), where samples were processed within 24 hr of receipt or kept at −80 C before processing. Enteric tissues were processed for further studies as follows: approximately 1 g intestinal tissue was homogenized with a fast-prep glass bead homogenizer (Savant Instruments, Holbrook, NY) in 0.5 ml sterile 50% Dulbecco's modified Eagle's medium and 50% F12 medium (Mediatech, Herndon, VA). Samples were clarified by centrifugation for 15 min at 15,000 × *g*. Supernatants were used for DNA extraction using DNeasy blood and tissue kit (Qiagen), including 5 μg of carrier RNA.

Development and evaluation of PCR procedure. DNA was extracted from individual chicken and turkey enteric samples as described above. The complete parvovirus NS gene was amplified from extracted DNA using a 50 μl reaction mix that contained 25 μl HotStar*Taq* DNA polymerase (Qiagen) and 20 pmol of the previously described NS gene-specific forward and reverse primer sets NSF1/NSMR1 and NSMF1/NSR1 in order to create overlapping segments (39). PCR was performed using a 15-min incubation at 95 C followed by 30 amplification cycles (94 C for 30 sec, 55 C for 1 min, and 68 C for 3 min). Three sets of overlapping amplicons from chicken samples, and three sets of overlapping amplicons from turkey samples representing six individual full-length NS gene sequences, were purified with a PCR purification kit (Qiagen) and eluted in 20 μl of the elution buffer (Tris-ethylenediaminetetraacetic acid) provided by the manufacturer. Five microliters of the eluted, purified PCR product were ligated to the vector TOPO-Blunt (Invitrogen, Carlsbad, CA) and used to transform *E. coli* TOP-10 cells (Invitrogen) according to the manufacturer's instruction. Bacteria were plated on Luria Bertani (LB) agar plates containing kanamycin at 50 μg/ml. Colonies were inoculated into LB broth (containing kanamycin) in 96-well plates and incubated at 37 C for 24 hr in a shaker incubator. DNA was prepared using a PerfectPrep Plasmid purification kit (Eppendorf North American, Westbury, NY), sequencing was performed with M13 forward and reverse primers, and the reaction products were separated on an AB-3730 automated DNA sequencer (Applied Biosystems Inc., Foster City, CA). The final DNA consensus sequence had an average eightfold redundancy at each base position.

The sequences of the parvovirus NS gene from the six enteric samples were aligned with the previously published chicken parvovirus and turkey parvovirus NS sequences (39) using the ClustalW program (35). PCR primers were designed from regions of the aligned sequence showing high levels of nucleotide conservation and were designated as PVF1 and PVR1. Primer PVF1 (5′-TTCTAATAACGATATCACT-CAAGTTTC-3′) was at position 1431–1457 nucleotide (nt) in the NS gene sequence while PVR1 (5′-TTTGCGCTTGCGGTGAAGTC-TGGCTCG-3′) was at position 1965–1991 nt. To test reaction conditions, extracted DNA from ABU parvovirus-inoculated chicken enteric homogenates (ABU DNA) was used as a template for PCR. The 50-μl reaction mix contained 25 μl HotStar *Taq* solution (Qiagen) and 20 pmol of each of the PVF1 forward and PVR1 reverse primers. After 15 min incubation at 95 C, 30 cycles of amplification (94 C for 30 sec, 55 C for 1 min, and 68 C for 1 min) were performed. PCR products

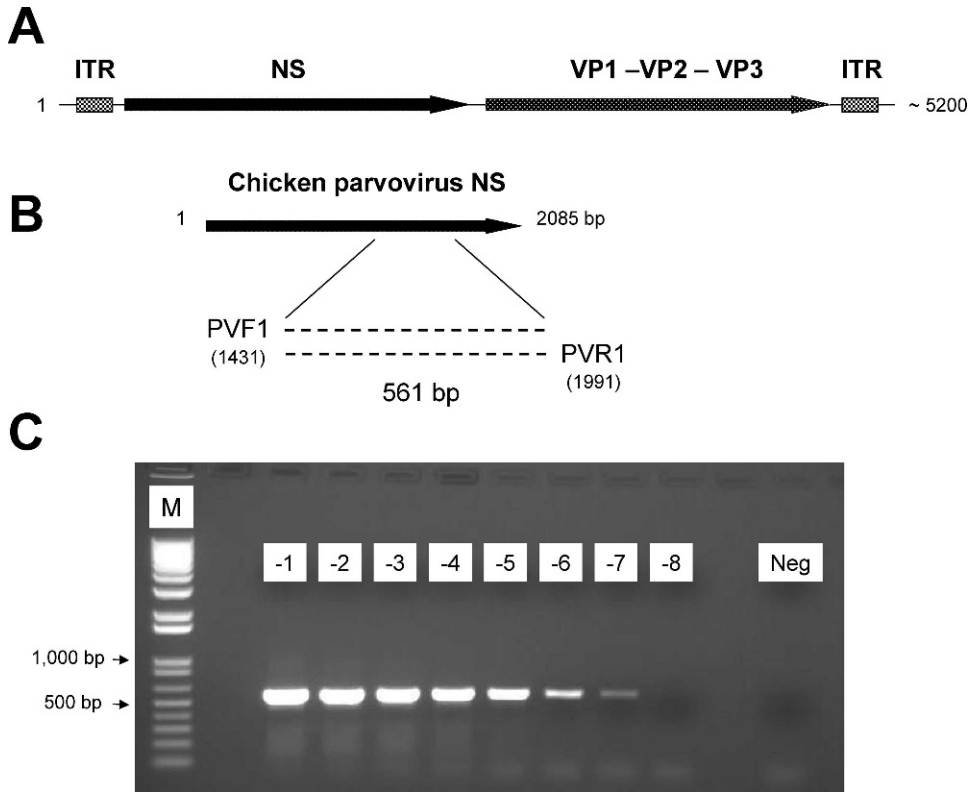

Fig. 1. Development of a PCR test to detect chicken and turkey parvoviruses. (A) The common genome structure of the autonomously replicating parvoviruses (6). ITR = inverted terminal repeat; NS = nonstructural gene; VP = structural viral protein. (B) Three chicken and 3 turkey parvovirus NS gene sequences were aligned, and highly conserved regions at nucleotide position 1431–1457 and 1965–1991 were used to design primers, PVF1 and PVR1, respectively. (C) Using these primers, approximately 100–500 viral DNA equimolecules could be detected by a conventional PCR assay with a cloned parvovirus NS gene fragment as target DNA.

were visualized after separation in an ethidium bromide stained agarose gel.

To test the sensitivity of the assay, a 1081-base pair (bp) PCR fragment was amplified from ABU DNA using primers NSMF1 and NSR1. The amplicon containing the PVF1 and PVR1 annealing sites was cloned into a TOPO-TA vector to create a 5012-bp double-stranded DNA product. DNA was purified by a miniprep DNA purification kit (Qiagen) and used as a target to determine the number of DNA molecules the PCR assay could detect. Tenfold serial dilutions of the target DNA were used, and PCR reactions containing the PVF1 and PVR1 primers were performed as described above. Assay sensitivity was calculated based on the DNA concentration of each dilution and on length of the template DNA molecules.

To determine the specificity of the PCR assay, target DNA was extracted from the following materials as described above: uninfected primary chicken embryo fibroblast (CEF) and chicken embryo liver (CEL) cell cultures; allantoic fluid of uninfected 9-day-old embryonating chicken eggs; TOPO-TA vector plasmid and *Escherichia coli*; enteric homogenates of uninfected SPF chickens; and enteric homogenates containing enteric viruses (astrovirus, reovirus, rotavirus, coronavirus, adenovirus) (21).

Phylogenetic analysis. PCR amplicons using primer sets PVF1 and PVR1 from selected parvovirus-positive field samples were used for subsequent phylogenetic analysis. A total of 38 positive PCR amplicons (using PVF1 and PVR1 primer sets) from field enteric samples were cloned and sequenced with M13 forward and reverse primers. Ten chicken and 10 turkey field parvovirus sequences were used to construct a phylogenetic tree. The amplicons were purified and ligated into the TOPO-TA cloning vector, and this was used to transform TOP-10 *E. coli* cells. DNA preparation and sequencing were performed as described above. Phylogenetic and molecular evolutionary analyses were conducted using MEGA Version 4.0 software package (32) (http://www.megasoftware.net/mega4).

RESULTS

Development of a conventional PCR to detect chicken and turkey parvoviruses. Based on multiple alignment data of three chicken and three turkey parvovirus NS gene sequences, two regions were found, at positions 1431–1457 nt and 1965–1991 nt within the NS gene, that showed a high level of nucleotide conservation (Fig. 1B). Using primers from those sequences, approximately 100–500 parvovirus DNA equimolecules could be detected by a conventional PCR assay (Fig. 1C). No parvovirus DNA was detected by PCR using template DNA extracted from uninfected CEF and CEL cells, allantoic fluid, vector plasmid, and *E. coli* and enteric homogenates containing astroviruses, reoviruses, rotaviruses, coronaviruses, or adenoviruses.

Evaluation of the PCR assay to detect parvovirus in cloacal swab and plasma samples of experimentally infected chickens. SPF chickens were experimentally infected with ABU parvovirus and cloacal swab samples and plasma samples were taken at various times postinfection. PCR analysis of these samples showed the presence of parvovirus as early as 4 and 7 DPI in cloacal swabs and plasma samples, respectively (Fig. 2). By 14 DPI, 100% of both swab and plasma samples became positive, and the virus persisted at a detectable level in infected birds until 28 DPI. No positive PCR amplification was observed with samples collected from uninfected SPF birds during the study.

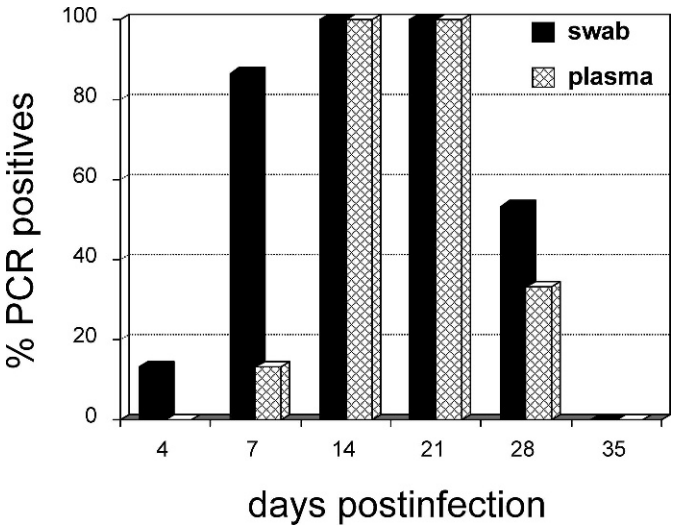

Fig. 2. Parvovirus in experimentally infected chickens. SPF chickens ($n = 15$) were experimentally infected with ABU parvovirus, then cloacal swab samples and plasma samples were taken from each bird at indicated times postinfection, and PCR analysis was performed to show the presence of parvovirus. Control birds were negative at each time point.

A total of 20 randomly selected PCR amplicons, amplified from cloacal swab and plasma samples using PVF1 and PVR1 primers, were cloned and sequenced. All of those sequences were identified as parvoviruses, and amplification of nonparvovirus DNA sequences was not observed in the study.

Prevalence of parvovirus infections in chicken and turkey flocks in the United States. A total of 138 samples from 10 different states were tested by PCR for parvovirus presence (Table 1). Of the chicken samples that were collected in 54 farms, 77% (46/60) were positive for parvovirus, while 78% (61/78) of the turkey samples that were received from 29 farms were parvovirus positive. Parvovirus infection was detected in bird samples from 8 different states including GA, SC, NC, DE, MO, AR, MN, and CA. There was no direct correlation between the age of the birds and virus presence. PCR-positive samples could be identified between 5 days and 8 wk of age in both chicken and turkey enteric samples.

Phylogenetic studies using chicken and turkey parvovirus NS gene sequences. Phylogenetic analysis revealed that the chicken and turkey parvoviruses clustered in separate groups, with the exceptions of the Ch841AR05 chicken virus that grouped with the turkey viruses and the Tu260CA05 turkey virus that grouped with the chicken parvoviruses (Fig. 3). The overall nucleotide sequence identity among chicken isolates was 95.4%–98.6%, while the turkey isolates revealed somewhat higher identities with each other, ranging within 99.3%–99.6%. There was a 90.0%–90.5% mean nucleotide sequence identity between the clustered chicken and turkey parvovirus NS gene regions.

Table 1. Parvovirus detection in enteric samples of commercial chicken and turkey flocks.

Species	State of origin (number of samples)	Date of collection	Number of parvovirus positives (%)
Chickens	GA (10)	2005, 2006	7
	NC (3)	2005	1
	SC (1)	2005	1
	DE (1)	2005	1
	MO (16)	2005, 2008	11
	AR (23)	2005, 2008	19
	CA (6)	2005, 2006	6
	Total (60)		46 (77)
Turkeys	NC (22)	2003, 2005, 2008	21
	VA (1)	2003	0
	MN (12)	2007	5
	TX (1)	2004	0
	CA (42)	2004	35
	Total (78)		61 (78)

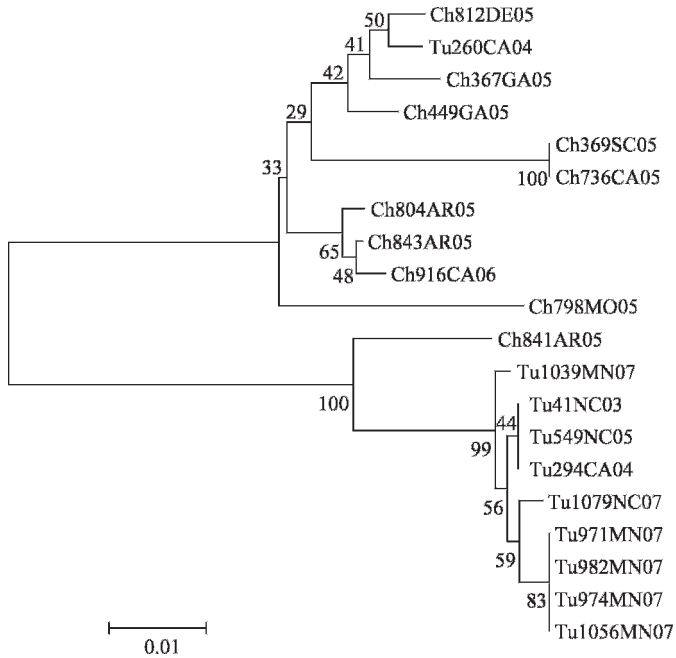

Fig. 3. Evolutionary relationships of parvoviruses detected in chicken and turkey enteric samples. A conserved sequence of 561 nt of the PCR amplicons was aligned to construct the phylogenetic tree. The evolutionary history was inferred using the neighbor-joining method (28). The optimal tree, with the sum of branch length = 0.19500136, is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (11). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method (33) and are in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA4 (32).

DISCUSSION

In this study, we describe the development and application of a conventional PCR assay to detect parvoviruses in poultry samples. Our data indicate that chicken and turkey parvoviruses are widely distributed in commercial poultry flocks in the United States, and that these viruses have a potential role in the etiology of enteric diseases in poultry.

Previously, we reported the application of a random molecular-screening method for the detection of novel parvoviruses in intestinal samples of chickens and turkeys exhibiting characteristic signs of enteric disease (39). Sequence analysis of the left end of the genome, including the complete NS gene, demonstrated that the chicken and turkey parvoviruses were closely related to each other and that they are representative of a novel member of the parvovirus family. Using electron microscopic examinations, parvoviruses have been identified

in the past in turkeys and chickens exhibiting enteric disease characterized by stunting, diarrhea, and mortality (17,18,36). However, the prevalence of parvoviruses in chicken and turkey flocks has not been further studied, mainly because of the lack of more-definitive methods to identify parvovirus from field samples.

Our previous data demonstrated a high level of homology between chicken and turkey parvovirus NS genes (39). In this study, further comparative sequence analysis of six independent parvovirus NS genes revealed specific regions within the NS gene with 100% nucleotide sequence identity. PCR primers were designed to target these highly conserved regions, and a conventional PCR assay was developed. In our studies, this PCR assay proved to be highly sensitive and specific to detecting parvoviruses in clinical samples from experimentally infected birds and field samples. For the first time, it is reasonable to conclude that this PCR assay should prove to be a fast and reliable tool for the diagnostics of parvovirus infection in poultry.

There is no information in the literature concerning the prevalence of parvovirus infection in commercial poultry flocks. Our survey clearly demonstrates that parvoviruses are widely distributed in both commercial chicken and turkey flocks in several states in the United States. Our data show that, in fact, most if not all of the major poultry producing states have poultry flocks where parvovirus infection was detected. Like other parvovirus infections (15), chicken and turkey parvoviruses were present in very young birds, as early as 4 days of age, confirming the possibility of a potential vertical transmission of the virus (16). Considering the rapid growth characteristics of parvoviruses (3) and their efficient spread via the fecal–oral route, horizontal transmission of the virus from infected birds to naïve individuals may also play an important role in the spread of virus infections within flocks. In conclusion, the high prevalence of parvovirus infection in young birds suggests a potential role of these viruses in the etiology of enteric disease of poultry.

Phylogenetic analyses comparing NS gene segments revealed a strong similarity between the chicken and turkey parvoviruses. It was also evident that most of the chicken and turkey parvovirus isolates formed distinct phylogenetic groups, suggesting that these viruses might have diverged from a common ancestor and subsequently went through a host-specific adaptation. A high level of sequence identity of the NS gene was reported between the Muscovy duck and goose parvoviruses (38). The duck and goose parvoviruses have a high level of sequence identity (81.9%) across their entire genome and have caused similar diseases in their respective hosts (13). Based on the strong genome sequence homology, it is tempting to speculate that the chicken and turkey parvoviruses could have similar, or potentially identical, pathogenic properties in their respective hosts.

Although our data suggest that parvovirus infection is highly prevalent in poultry flocks affected by enteric disease, there was no definite correlation between virus presence and disease. This is not surprising, because it has been well documented with other parvovirus infections, including goose parvovirus and human boca parvoviruses, that parvoviruses or their DNA can be frequently detected from both healthy and diseased individuals, especially at an early age of their life (13,15). Importantly, as was shown in goose parvovirus infection, maternally acquired virus-specific antibodies play a significant role in the epidemiology of clinical disease, and the level of passive immunity determines the susceptibility of the progeny following virus infection (13).

It is clear that in order to define the exact role of parvoviruses in the etiology of enteric diseases of poultry, a complex sero-epidemiologic study will be necessary. One of the major obstacles to studying the epidemiology and biology of chicken and turkey parvoviruses is the lack of reagents, specifically *in vitro*-propagated infectious virus and antiparvovirus antibodies. Our future studies will focus on the complete sequence determination of these novel parvovirus genomes and on production of specific reagents to develop sensitive serologic assays.

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