PATHOGENICITY, ANTIGENICITY,
AND DETECTION OF TURKEY ASTROVIRUSES

DISSERTATION

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ABSTRACT

Astroviruses are etiologic agents of acute gastroenteritis in human beings and in a variety of animal species. Turkey astrovirus is an important pathogen of poult enteritis and mortality syndrome (PEMS), which has caused significant losses to turkey industry in the USA. Unlike the situation of human astroviruses, turkey astroviruses have not been well characterized in terms of pathogenicity, and antigenicity. In the first part of this study, a pathogenicity investigation was carried out in specific pathogen free (SPF) turkey embryos and commercial poults. Turkey astrovirus 1987 (TAstV1987) isolate and turkey astrovirus 2001 (TAstV2001) isolate were used in this investigation. Both virus isolates caused gastroenteritis and growth depression in both SPF turkey embryos and commercial poults. The histopathologic lesions on thymus and bursa induced by TAstV2001 in embryos and poults were not remarkable. Similarly, no abnormal morphologic changes were noted on thymus of embryos and poults inoculated with TAstV1987. There was no statistically significant difference in bursa weight / body weight ratios (P>0.05). However, TAstV1987 infection resulted in microscopic lesions of infected bursas of embryos and poults.

In the second part of this study, The antigenicity of TAstV1987 and TAstV2001 were compared with each other, and the antigenic relatedness were determined by cross-neutralization tests in turkey embryos as well as by enzyme-linked immunosorbent assay.
The antigenic relatedness values (R) were calculated using the Archetti and Horsfall formula. The R value as measured by cross-neutralization tests was 0.56%, indicating the TAstV1987 and TAstV2001 belong to different serogroups; whereas the R value measured by ELISA was 70.7%, suggesting these two viruses share common antigen(s).

So far, the diagnosis of astroviruses was mainly dependent on electron microscopy (EM) or immune electron microscopy (IEM) techniques. To develop other diagnostic assays, an antigen capture enzyme-linked immunosorbent assay (AC-ELISA) was developed using polyclonal hyperimmune antisera against TAstV1987 and TAstV2001 isolates, and monoclonal antibody (MAb) for TAstV2001. Monoplex and multiplex reverse transcription /polymerase chain reactions (RT-PCR) were developed as well using non-degenerate primer sets specific to the capsid region, and degenerate primer pairs covering the polymerase area of turkey astrovirus genome. One ssRNA internal control (IC) template reagent was produced, and applied to the RT-PCR to reduce the false negative percentage of the test. Direct EM was used also in this study and the results obtained from both EM and RT-PCR were compared. The results showed that the polyclonal AC-ELISA had higher sensitivity and a wider detection spectrum than that of the monoclonal AC-ELISA with group-specific MAb, but with higher background, whereas the monoclonal AC-ELISA had very high specificity but lower sensitivity (0.06 µg of viral proteins). Fecal samples or gut content samples from naturally infected or experimentally infected poult with PEMS were examined by EM and RT-PCR. The EM results indicated that the positive percentage of small round viruses (SRVs) that could include astroviruses as well as enteroviruses was 33.4%. The
monoplex RT-PCR results amplified with primers SRV-1-3 and SRV-1-5 revealed that the positive percentage of astroviruses was 45.3%, which is 10.9% higher than that of direct EM even if other SRVs were not excluded. Multiplex RT-PCR with both SRV-1-3 and SRV-1-5 and AFCP-F1 and AFCP-R1 and the monoplex RT-PCR with degenerate primers showed good specificity and wider detection spectrum than that of earlier published data and the monoplex RT-PCR with the positive percentage of 59.4%, 25% higher than that of EM. With the internal control, an overall test inhibition rate of 12.5% was found for the astrovirus RT-PCR assay, which can be used to decrease the false-negative percentage of the detection.
Dedicated to my wife Guangyu Liu,

my son Hong, and my mom
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CHAPTER 1

LITERATURE REVIEW:
HUMAN AND ANIMAL ASTROVIRUSES

1.1 Introduction

Astroviruses are etiologic agents of acute gastroenteritis in children (3, 76, 88, 89) and young animals including sheep, cattle, swine, dogs, turkeys, cats, deer, mice, and minks (18, 32, 53, 62, 91, 92, 99, 132, 133, 135, 136, 142, 148, 158, 160). In addition to causing intestinal illness, extraintestinal illness was also noted in some avian species. For example, astroviruses cause a rapidly fatal hepatitis in ducklings with mortality rates of up to 50% (40), and cause growth retardation and acute interstitial nephritis in chickens (55). Human astroviruses (HAstVs) infections have been found worldwide, and identified as the second most important cause only to rotavirus of viral infantile diarrhea (13, 17, 94). Human astroviruses were well characterized in terms of etiology, antigenicity, serology, distribution, and transmission. Turkey astroviruses (TAstVs) have been incriminated as an etiologic agent of enteric disease and turkey poult enteritis and mortality syndrome (PEMS), a highly pathogenic multi-system disease (99, 132, 135). TAstV infections are geographically widespread (131). In a surveys of commercial
turkey flocks with enteric disease, astroviruses were either the most commonly detected viruses or the second most commonly detected viruses (134). In the eighties, all the astroviruses detected were strictly confined to the gastrointestinal (GI) tract of turkey poult's and the experimental disease they initiated was an enteric disease and no evidence of a systemic disease was detected. In the nineties, TAstV was found not only in the intestines, but also in thymus, and bursa, suggesting a possible immunosuppressive role related to PEMS. The economic impact of PEMS was well documented (7). However the exact role of astroviruses in PEMS is not clear yet (66, 67, 164).

1.1.1 History

Astrovirus was first described by Madeley & Cosgrove in 1975 (88) in infantile viral gastroenteritis. They observed small round viruses with a five- or six-pointed star-like appearance by direct electron microscopy (EM). Hence they named them astroviruses based on the distinctive surface features of these small round viral particles. However, this was not the first report about astrovirus. Actually in an earlier report in the same year (1975), Appleton and Higgins (3) found these small round virons with 29 to 30 nm in diameter in the stools of infants hospitalized with mild diarrhea, and vomiting. These viruses ware different from the previously reported rotavirus and Norwalk virus in morphology and size, and these viruses did not show the astrovirus-like capsid profiles. In 1982, Caul E. O., and Appleton (21) eventually demonstrated these viruses to be astroviruses when immunologic reagents were available. Afterwards, astroviruses were identified in association with gastroenteritis in a wide range of species, such as lambs.
Turkey astrovirus was first reported in poults associated with diarrhea and increased mortality by McNulty et al in UK (1980) (99). Subsequently, Saif, et al., and Reynolds et al. identified this virus in the US.(132, 133, 135, 136). In a survey, Reynolds et al. (134) found astroviruses in 78% of diseased turkey flocks, being the most frequently detected enteric viruses. Later on astroviruses were shown to be widely distributed in commercial turkey poults, and an important etiologic agent in PEMS (131).

Duck astoviruses and avian nephritis viruses. Unlike other astroviruses species, which are usually associated with gastroenteritis, duck astrovirus, known as duck hepatitis virus type II (DHV type II) historically, causes a fatal hepatitis in ducklings, and avian nephritis viruses (ANV) cause nephritis in chickens. Both viruses were classified into Picornaviridae. In 1984, 2000 and 2001, DHV II, ANV-1, and ANV-II were re-classified as members of the family Astroviridae based on their genome sequence, respectively (40, 55).

1.1.2 Etiology

1.1.2.1 Classification. It was not until 1993, that astroviruses were classified into a new family of RNA viruses, Astroviridae (110). Before that, they were classified as members of Picornaviridae since they had similar morphology and size when visualized under EM, or immune electron microscopy (IEM). However the molecular structures or the replication mechanism of astroviruses differ from picornaviruses and caliciviruses, which are well characterized two families of nonenveloped, monopartte positive RNA
viruses. A common feature of replication of those positive sense single stranded RNA viruses during their life cycle is to produce one or more subgenomic mRNAs encoding the structural proteins. Rather, Picornaviruses do not synthesize any subgenomic RNAs. Caliciviruses do produce subgenomic mRNAs when replicating, however their capsid protein profile is different from astroviruses, the former contains only one capsid protein of 58 – 65 kd, instead of that, the latter, astroviruses both from humans and from animals consist of several capsid proteins in a variety of size (20, 95, 156). Moreover, astroviruses lack of a helicase domain, and utilize ribosomal frameshifting mechanism to translate the RNA-dependent RNA polymerase. All of these incorporate specific features of astroviruses distinguish them from any other group of viruses. Therefore, astroviruses were assigned to a separate new family, Astroviridea, of nonenveloped RNA viruses in the Sixth Report of the International Committee on the Taxonomy of Viruses (ICTV) (107).

1.1.2.2 Morphology

Astroviruses are non-enveloped small round RNA viruses with a simple construction. The capsid is 27-30 nm in diameter and the capsid/nucleocapsid exhibits polyhedral symmetry. The capsomer arrangement is clearly visible. Surface spikes are small and surface appears rough. External diameter (with spikes) is approximately 43 nm. Around 10% of virions of astroviruses when examined by EM display a typical five- or six-pointed star-like appearances which led to the naming of this agent. The star-like morphology feature is pH dependent. After alkaline treatment (pH10), the starlike feature was inducible in negatively stained preparations. The viral particles in high pH
 (>10) are subject to disruption. In most cases with common preparations of fecally shed viruses, the viral surface structure is featureless. One cannot differentiate astroviruses from other small round viruses, such as enterovirus, parvovirus, parvovirus-like viruses only by the capsid morphology (164). Although IEM can be used to enhance the specificity and sensitivity of detection of astroviruses, it may further obscure some of the surface features (5, 70, 72). In some preparations, “bridging structures” between the adjacent astrovirus virions have been observed. These structures may be the surface extensions of the virus (53, 87, 88). However, the significance of these structures remains to be elucidated (95).

### 1.1.2.3 Physicochemical properties

A range of buoyant densities in cesium chloride (CsCl) was reported for human and animal astroviruses (95). The density of intact human astrovirus virions was shown to be 1.35 to 1.37 g/ml (21, 72, 96, 154). Whereas, the buoyant density of turkey astrovirus was between 1.34 to 1.36 g/ml (164). The documented highest density of astroviruses is found to be a range of 1.39 to 1.40 g/ml (68, 101). Both human astroviruses and turkey astroviruses are extremely stable to inactivation. Astroviruses are stable at pH 3 and resistant to heat treatment, chloroform treatment, a variety of detergents and lipid solvents, as well as most alcohols except 90% methanol (72, 164). Disinfection of astroviruses under laboratory conditions can be accomplished by using 90% methanol or peroxymonosulfate-containing disinfectants (139).
1.1.2.4 Genomic Organization and Viral Proteins

To date, ten complete genome sequences of astroviruses are available in the database. Half of them are from human astroviruses. They are: (a) the serotype 1 Oxford reference strain propagated in LLCMK2 cells (85), (b) a serotype 1 Newcastle strain isolated and maintained in CaCo-2 cells (151-153), (c) the serotype 2 Oxford reference strain propagated in LLCMK2 cells (56), (d) a serotype 3 strain (GenBank accession No. AF141381), and (e) a serotype 8 strain (GenBank accession No. AF260508). The rest of the five genomic sequences are available from other species: sheep astrovirus (GenBank accession No. Y15937), turkey astrovirus 1 (GenBank accession No. Y15936), turkey astrovirus 2 (67), avian nephritis virus (55), and mink astrovirus (GenBank accession No. AY179509).

The genome of astroviruses is a plus sense, single stranded RNA with 6.8 kb to 7.5 kb nucleotides in length. The 5´-end of RNA is linked covalently to a protein, Vpg, and the 3´-end is polyadenylated. The number of proteins in virions is not clear. There are two major, several minor capsid proteins, and nonstructural proteins (95).

The genomic organization of astroviruses from humans and animals are similar. They all consist of three open reading frames (ORFs), i.e., ORF-1a, ORF-1b and ORF-2 in order of 5´ end to 3´ end. For human astroviruses, a 5´-untranslated region (UTR) with around 80 nt precedes ORF-1a. Similarly, there is about 80 nt untranslated region between ORF-2 and the poly (A) tail at the 3’end of the genome. However, the corresponding areas of avian astroviruses vary to some extent. Generally, the ORF-1a encodes nonstructural proteins, ORF-1b encodes an RNA dependent RNA polymerase, and ORF-2 encodes the structural proteins, that is, capsid proteins precursors. Some
ORFs of human astroviruses were well characterized. The ORF-1a of human astrovirus serotype 1 contains a transmembrane helices protease motif, a putative protease-dependent cleavage site, a nuclear localization signal (NLS), a ribosomal frameshifting signal, and an immunoreactive epitope (96). The ORF-1b possesses an RNA polymerase motif, and the first in-frame start codon AUG was found in that motif. ORF-2 encodes an 87 kd capsid protein precursor that will be cleaved into several capsid proteins (8), and the 87 kd of the precursor can be utilized to assemble an intact viral-like particle (VLP) in vitro, which may be used as a candidate vaccine of astroviruses in the future (29). The half ORF-2 of N-terminal exist a highly conserved area. There is a short overlap region between ORF-1a and ORF-1b, as well as ORF-1b and ORF-2. The ORF-1a/ORF-1b overlap region possesses a heptameric shift sequence which would potentially form a downstream stem-loop and possible pseudoknot involving a ribosomal shifting mechanism when translating (55, 67, 86, 151). The ORF-2 is found in both the genomic and subgenomic RNA, The role of subgenomic is the production of viral capsid proteins (110, 112).

The genomes of animal astroviruses have some unique characteristics that distinguished from other positive-sense, single-stranded RNA viruses, that is, they use ribosomal frameshifting mechanism to express the RNA polymerase, and possess a serine protease.

Information on expression and processing of nonstructural proteins of astroviruses is limited, there are some conflicting reports (37, 59, 150).

The capsid proteins profiles of ovine, porcine, turkey, and several serotypes of human astrovirus have been reported. The results from those reports did not concur on
the size and number of structural proteins comprising astrovirus particles. For lamb astrovirus, two 33 kDa polypeptides were described (48). Study on porcine astrovirus revealed five structural proteins with mass that range from 13 to 39 kDa has been identified (141). Kurtz et al. found there were four proteins with mass of 36.5 kDa, 34 kDa, 33 kDa, and 32 kDa in astrovirus serotype 4 propagated in cell culture in the presence of trypsin (72), and two additional (24 kDa and 5.2 kDa) in serotype 1 astrovirus (71). Sanchez-Fauquier et al. demonstrated that an 86 kDa protein is the precursor of VP26, a major component of virions by using neutralizing monoclonal antibody (PL_2) (138). VP 29 (a minor protein of virons), the product of alternative processing of P86, was also identified. An analysis of turkey astrovirus capsid proteins using SDS-PAGE and Western blot revealed 3 polypeptides with molecular weight of 34.5, 31, and 28 kDa (164). Bass and Qiu (8) recently characterized the nature of proteolytic processing of the astrovirus capsid. They found that an approximately 87 kDa capsid protein was initially translated, then converted to 79 kDa form, which can assemble into viral capsid, but noninfectious, it turns infectious with conversion of the 79 kDa capsid protein to three smaller peptides of 34, 29, and 26 kDa.

1.2 Strain Classification and Antigenicity

Eight serotypes of human astroviruses were identified by various methods. Five of them were differentiated by immunofluorescence, neutralization, and IEM (54, 73, 79). Serotype 6 and 7 were indistinguishable from serotype 1 to 5 by EM (27, 80). The eighth serotype was identified by immunofluorescence assay (12). In general, astrovirus serotype 1 is the most frequent type found (35, 58, 72, 73, 80, 113, 114, 116, 117, 121, 137, 159),
However the predominant serotype may vary depending on geographic area (45). Hyperimmune antisera derived from animals against the human astroviruses serotypes 1 to 7 react in a serotype-specific manner in IEM and immunofluorescence assays (50, 72, 80). In addition, Herrmann J. E., et al. found polyclonal antiserum from rabbit neutralized astrovirus serotype-specifically in a plaque assay (50). They also demonstrated a group specific monoclonal antibody (MAb), a non-neutralizing MAb, that can recognize all eight serotypes of human astroviruses (51, 58, 80). There was a good correlation between serotypes and genotypes based on the nucleotide sequence analysis of a limited region of ORF2 of human astrovirus genomes (10, 117).

Two or three serotypes of bovine astrovirus (108) and one serotype of porcine astrovirus (111) have been identified. Antigenenically, human, lamb, piglet, red deer, kitten, calf, duckling, chick, and turkey poult astroviruses are unrelated to each other. The astrovirus infection is a strictly species specific, and there is no evidence of cross-reactivity between species (18, 47, 79, 148, 162). For example, convalescent sera from red deer infected with deer astrovirus did not recognize lamb astrovirus antigens in infected lamb intestinal tissue and vise versa (95).

Turkey astroviruses have not been serotyped. There are two genotypes of turkey astroviruses (TAstV-1 and TAstV-2) whose complete genome sequence data are available in GenBank (accession No. Y15936, and accession No. AF206663). TAstV-2 is genetically and immunologically distinct from TAstV-1 (67).
1.3 Virus replication

Information regarding the virus replication is very limited. In general, turkey astrovirus replicates in the apical sides of enterocytes along the sides of the villi, but rarely in crypts. Viral particles aggregated into crystalline array as well as free viral particles were observed within enterocytes in ultra-section sample by EM (131).

1.4 Diagnosis and Pathology

1.4.1 Disease diagnosis

Until recently, the primary method to detect astroviruses in stool, or feces/gut contents was direct EM with negative staining. This conventional method is relatively sensitive enough to identify the agents since the patients or animals with diarrhea caused by astroviruses frequently shed large numbers of viral particles (72). The sensitivity of direct EM has been estimated to be $10^6$ to $10^7$ virus particles per gram of specimen (95). However, as mentioned before, only 10% of viral particles may exhibit the five- or six-pointed star-like characteristic, so it is difficult to differentiate astroviruses from other surface featureless small round viruses. Due to this limitation of EM, Reynolds (130) and others suggested the use of IEM techniques which can enhance not only the specificity of the test but also the sensitivity if the corresponding antiserum available (14, 61, 68, 130), although it may mask the characteristic morphologic feature or fail to detect new serotypes.

An antigen capture EIA for detecting human astroviruses was developed by Herrmann et al. using a group-specific MAb (8E7) (50, 51). A modification of this assay using biotinylated detector antibody was established by Moe C.L., et al. (105). Both

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EIAs were shown to be useful tools, especially when a large numbers of samples need to be tested during epidemiologic investigation (26, 52, 83, 105, 140). However the sensitivity of EIA is not very high, similar to IEM (10^5 to 10^6 viral particles per gram of stool) (51, 105). For turkey astrovirus detection, Koci et al. (64) analyzed the reactivity between a recombinant TAstV-2 capsid protein and a MAb against TAstV (produced by James Guy (46), North Carolina State University) by western blot, enzyme-linked immunosorbent assay, and immunofluorescence in transfected cells (unpublished data). They found that all tests were positive.

In addition to the above phenotyping methods, genotyping assays, such as molecular probe and reverse-transcriptase polymerase chain reaction (RT-PCR), were recently developed to detect both human astrovirus and turkey astrovirus as the partial or complete gene or genomic sequence data became available (57, 65, 105, 155, 157). The sensitivity of probes is similar to EIA (105, 155). But the sensitivity for RT-PCR is much higher compared to EIA. It was estimated to be 10 to 100 viral particles per gram of sample (137). For example, RT-PCR detected astrovirus in 32% of the samples tested, whereas EIA only had a detection rate of 10% in an investigation of an outbreak of astrovirus gastroenteritis at a day care center (103). At present, the more and more sequence data have become available, enable to design primer sets which can be used to identify not only multi-serotypes but also can differential specific strains. For this purpose, Koci et al. (65) tried to design two sets of primers, one based a conserved polymerase region, and one from variable region. For human astrovirus detection, a pan-reactive RT-PCR was developed, which had a good correlation with EIA. Unfortunately,
this kind of test could not be developed for animal astrovirus identification since the similarity within/between the species of animal astroviruses is not high.

Cell culture is a conventional but useful technique for the detection of human astroviruses in clinical specimens. Human astroviruses can be propagated in certain specific cell lines, such as CaCo-2, T84, HT-29, and MA-104, whereas Norwalk virus and human caliciviruses remain refractory to growth in this system. Based on the growth property, people can distinguish astroviruses from other enteric viruses (19, 81, 144). Of these techniques, Shell vial IFA, a combination of cell culture and immunofluorescent antibody test, is more practical for clinical laboratories (19).

1.4.2 Morbidity and Mortality

Human astrovirus infections are well documented worldwide. Generally the infections have been associated with epidemic gastroenteritis in institutions with infants or the elderly and also with endemic illness in young children, in particular, under ages of five (3, 5, 6, 9, 25, 26, 28, 30, 33, 34, 39, 41, 43, 44, 52, 68, 69, 76, 82-84, 89, 90, 98, 100, 102, 104, 105, 109, 114). In one study from the public health laboratory surveillance system in the United Kingdom it has shown that astrovirus infection rate is as high as 3% of all EM detections from 1985 to 1987 (109). Antibody prevalence surveys revealed that antibodies were present in 7% of infants less than one year of age, but in 70% of children by the age of 5 years (72). Several reports associated astroviruses with immunocomprised adults who have diarrhea. But the accurate incidence of astrovirus gastroenteritis is difficult to ascertain (95). Astrovirus gastroenteritis is usually mild, self-limiting illness which does not require hospitalization.
Turkey astroviruses usually affect 1 – 5 weeks old poults (132-134), and cause intestinal or/and extraintestinal illness. The morbidity caused by turkey astrovirus is as high as 100% (126). The results from one survey showed that astrovirus infection made up nearly 80% of affected flocks with diarrhea, which is the most prevalent enteric virus detected (134). Astroviruses have often been detected with other enteric viruses, especially group D rotavirus, and also in normal healthy birds (< 30%) (134). The mortality caused by astrovirus is usually low, but can be as high as 50% when coinfectected with other viruses (126). Thus, the high morbidity is of the greatest concern in poultry industry because the disease retards the growth rate of poults and lowers the feed conversion rate.

1.4.3 Clinical Features and Lesions

Human astroviruses (HAstVs) primarily affect young children aged < 5 years, most under one year of age (114), and cause gastroenteritis all over the world. HAstVs can also infect young adults, elderly institutionalized patients (41, 84, 97, 159), and persons infected with the human immunodeficiency virus (HIV) (44). They have also been implicated as a possible cause of a persistent diarrhea (149). The incubation period of astrovirus infection is 3 to 4 days. The illness induced by astroviruses is characterized by a mild, watery diarrhea, associated with fever, headache, malaise and occasional vomiting, abdominal pain, and the disease typically lasts for 2 to 3 days with some exceptions. The virus excretion can persist for up to 12 days (16, 42, 70, 72, 77, 115, 122). The clinical features of diarrhea caused by astrovirus and rotavirus in children are very similar, so it is difficult to be differentiated from each other on clinical grounds.
alone (24, 52, 72). Gastroenteritis caused by astroviruses is usually mild, self-limiting, and does not need hospitalization or specific therapy. In severe cases, e.g., becoming dehydrated, oral or intravenous fluid resuscitation may be necessary (95). No reports specifically addressed macroscopic lesions and microscopic lesions in human astrovirus infection.

As mentioned previously, astroviruses have been identified in association with diarrhea in several animal species. The infection is species specific. There is no evidence showing cross-reactivity between species (18, 47, 79, 148, 162). The real role of each astrovirus in the disease is not clear since the viral diarrheic disease is usually caused by a co-infection of multiple viruses in a nature infection situation. The data from experimental infection with each astrovirus alone, separated from other enteric viruses in diarrheic feces, showed the virus to cause no illness or mild illness (47, 141, 160-162). However combined infections using astrovirus and other causative enteric viruses resulted in severe diarrhea (162).

Likewise, in naturally infected turkey poults, TAstV and group D rotavirus were detected concurrently (133). Experimental studies have demonstrated that TAstV alone can induce enteric disease in SPF or naïve turkey poults (132, 139, 145). Turkey poults inoculated orally with TAstV developed diarrhea on 2 day post-inoculation (DPI), and persisted for 9 days (131). The disease was characterized by watery to frothy, yellow to-brown droppings, and stunted growth. The gross lesions consisted of dilated ceca containing yellow, frothy, gaseous intestinal contents, loss of intestinal tone, and hyperemia of the intestinal tract (133). Histopathologic lesions of mild crypt hyperplasia in the small intestine were observed by light microscopy. This change had showed up as
early as 1 DPI, and persisted through day 7. Intracytoplasmic aggregates of astovirus particles, arranging into either crystalline array or scattered, have been seen by EM on 2 DPI (145).

1.5 Virus Culture and Propagation

1.5.1 Propagation of Human astroviruses

Human astroviruses were first identified in fecal samples from infants with gastroenteritis using EM in 1975 (3, 88). Two years later, astrovirus replication was detected in infected primary human kidney (HEK) cells using immunofluorescent antibody (IFA) techniques (78). Until 1981, the serial passage of astrovirus in primary HEK became successful with the aid of trypsin (81). Then the astrovirus was successfully transfected in primary baboon kidney (PBK) cells and rhesus monkey kidney cell line (LLCMK2). This is an important progress in virus isolation and propagation (95). In 1984, five serotypes of human astroviruses were successfully propagated in LLCMK2 cells (73). At this time, cytopathic effects (CPEs) were not shown in the infected LLCMK2 cells. The infection of LLCMK2 inoculated directly with fecal filtrates containing astrovirus was not successful. Later, astrovirus serotype 1 from a stool sample was propagated on a continuous cell line of human colon carcinoma (CaCo-2) cells (154). An important development in this study was the direct isolation of HAstV with viewable CPE of infected cells. Afterwards astroviruses were propagated in 293 cells, an adenovirus transformed human embryonic kidney cell line, BHK-21 cells, PLC/PRF/5, a hepatoma cell line, respectively (31, 36, 93, 144). Up to date, all seven serotypes of human astroviruses can be propagated in cell lines CaCo-2, T84, HT-29, and as well as
MA-104, an African green monkey kidney cell line, Coupling cell culture with IFA staining technique, a shell vial assay for detection of human astroviruses was developed by Brinker et al., and the test is a rapid, sensitive and especially suitable for clinical laboratories (19).

1.5.2 Propagation of animal astroviruses

Unlike the progress achieved in human astrovirus propagation, attempts to adapt animal astroviruses in either primary or continuous cell lines were unsuccessful with the exception of bovine and porcine astroviruses (4, 141). Yu et al. tried to propagate a turkey astrovirus isolate in various cells, including turkey embryo kidney, turkey kidney, CaCo-2, Vero, and BGM-70 cells, but unfortunately, none of these cells supported the virus propagation (163, 164). Currently, the only laboratory host for TAstVs growth is turkey embryonated eggs (65, 67, 163, 164). TAstVs can be passed serially by inoculating 22-day-old SPF turkey embryos via the yolk sac or the amniotic cavity of 24 days old embryonated turkey eggs. On 4 or 5 DPI, the intestines as well as thymus and bursa from infected embryos can be harvested. The gross lesions with the distended, thin-walled, gaseous or fluid–filled intestines can typically be seen in the majority of infected embryos. But the infection does not cause mortality. The gut contents/fluid, the entire intestines, thymus, and bursa can be subsequently used for virus isolation and purification (65, 67, 163, 164). Two weeks old SPF turkey poults can also be used for turkey astrovirus propagation, but the replication of viruses in this system is not so productive as that in turkey embryos.
1.6 Immunity

1.6.1 Immunity to Human Astroviruses

There is limited information about the immunity to astrovirus. The determinants of immunity of astroviruses are not well recognized (95). One study demonstrated that the specific antibodies against astrovirus were mounted when rabbits were inoculated orally with astrovirus extracted from human feces. The antibodies can aggregate the homologous virus in IEM. The rabbits immunized with astrovirus did not develop illness, and not shed viruses when challenged with the same virus as in inoculum (63). The fact that primary infection provided immunity from severe disease on reinfection, were describes by Glass et al. (38). Similarly, studies carried in volunteers showed that persons with serum antibody to astrovirus did not produce diarrheic illness when challenged (74) Intravenous antibody therapy trial administered in one severe and astrovirus persistent gastroenteritis patient was shown to be very effective. The patient recovered, and viruses were non-detectable in the stools after 4 weeks (15). Immunological characterization of Marin County virus (MCV) has been completed by Herrmann and his colleagues (49). The results from this study revealed that rabbit anti-serotype 5 astrovirus antiseraum could cluster MCVs when examined by IEM. A group specific MAb can also recognize both prototype serotype 5 astrovirus and MCV. Moreover, “acute and convalescent sera from infected patients showed seroconversion to cell-propagated MCV by EIA” (49). Type 5 antiseraum, but not the MAb used in the study, can neutralize both reference type 5 and MCV in plaque-reduction assays. Naficy et al. (114) demonstrated that there was homotypic, but not heterotypic, immunity among the children infected with astrovirus. The fact that symptomatic astrovirus
infection occurring primarily in young children and elderly institutionalized patients suggests that antibody acquired early in life provides protection from disease (95).

In addition to systemic immune responses, the local mucosal immune system may also play an important role in defense of astrovirus re-infection (106). The human astrovirus–specific CD4+ T cells were found to lie in the intestinal lamina propria of adults.

1.6.2 Immunity to Animal Astroviruses

Immunity to animal astroviruses is not well documented. A study carried out in embryonic swine kidney cells showed that cells infected with porcine astrovirus did not produce CPE when the virus was incubated with convalescent serum from infected pigs (141), suggesting there was a specific astrovirus neutralizing antibody in convalescent serum. There is no report to specifically discuss avian astrovirus immunity, and related articles are limited too. Reynolds et al. (1986) found that SPF poults infected with astroviruses produced astrovirus-specific antibodies in their convalescent sera (132), which could aggregate astrovirus virions in IEM, and the birds stopped shedding viruses by 14 DPI. However, the role of convalescent sera in immunity against astrovirus re-infection is unknown, and the effect of maternal antibodies on infection is not clear either (132). As described previously, PEMS is an infectious, transmissible disease affecting young turkeys between 1-5 weeks of age. This disease is characterized by diarrhea, anorexia, growth depression, mortality and immunosuppression (7). TAstVs are important etiologic agent in PEMS (67, 128, 129, 163, 164). It was observed that TAstVs cause infection of not only intestinal tissue but also central and peripheral lymphoid organs in infected poults (126). In this study, the cell–mediated immunity, including T-
cell lymphoproliferative response and the constitution of some lymphocyte subsets, was examined. The findings from this study indicated that TAvS infection may compromise the lymphocyte-mediated immune defenses of infected birds (126). Furthermore, the TAvS infection also impairs the native immune system functions of affected poults in PEMS by inducing defects in macrophages, leading to increased susceptibility of poults to other agents (127).

1.7 Pathogenesis and Epidemiology

1.7.1 Pathogenesis

Pathogenesis studies on astrovirus infections in humans are not well documented. One of the reasons is the lack of a suitable animal model; another is that astrovirus infection is often a co-infection with other virus(es) or infectious agents. Thus it is difficult to determine the real role of astrovirus in the disease. A brief report suggested that human astrovirus replicated within small intestinal tissue (123). Another study demonstrated that astrovirus particles were localized to the intestinal epithelial cells in the biopsy specimens from patients suffering from gastrointestinal problems caused by either co-infection or some digestive enzyme deficiency (95). Therefore, the significance of this study is hard to assess. Kurtz and Midthun et al. (74, 101) carried out investigations of astrovirus infection in volunteers, but they did not conduct histopathologic examinations on those patients with diarrhea. Studies of viral pathogenesis in cell culture have been employed by some investigators (4, 31, 143, 154, 156). Some valuable findings have been achieved. However, some contradicting results were also obtained, for example, regarding the attaching and penetrating mechanism of virus, astroviruses are
likely to enter cells through the apical surface in polarized CaCo-2 cells, whereas, the wild type astrovirus seems to enter cells through the opposite direction, i.e., the basolateral surface (154). In fact, the findings obtained from *in vitro* studies do not always explain the *in vivo* mechanism.

By contrast, viral pathogenesis has been studied more extensively in animals. The specific-pathogen-free (SPF) turkey poult inoculated orally with TAstV alone produced diarrhea on 2 DPI, and persisted up to 7-9 days. The diarrheic disease is characterized by watery, frothy, yellow-brown droppings and growth depression (131, 146). The ability to absorb D-xylose was significant less compared with normal control birds. Specific maltase activity (SMA), a reflection of intestinal disaccharidase activity, of commercial turkey poult infected with TAstV decreased dramatically during acute infection period, but the change of SMA was transient and returned to normal level with resolution of symptoms by day 10 (146), suggesting the pathogenesis of diarrhea associated with astrovirus infection has been attributed to the osmotic effect of disaccharide maldigestion, malabsorption (146). Astrovirus infection of poult induces not only clinical signs and gross pathologic change, but also histopathologic lesions in intestines as described previously. A study of In-site hybridization using an RNA probe specific to one strain of TAstV has localized the astroviruses to the cytoplasm of enterocytes along the sides of villi (131). Ultrastructural data, mentioned above, have confirmed this observation. Generally speaking, astrovirus replicates in the enterocytes, then, the virus particles are released into intestinal lumen with enterocyte degeneration, which may also attribute to diarrhea too.
1.7.2 Distribution

The distribution of astroviruses has been demonstrated worldwide.

1.7.3 Natural hosts and transmission

As mentioned above, astroviruses can infect human beings as well as many other animal species. Human astroviruses affect primarily, but not exclusively, young children aged < 5 years old. Animal astroviruses only affect young animals. The astrovirus infection is species specific. There is no evidence that astroviruses could cross species (18, 47, 79, 148, 162). The mode of transmission for astrovirus infections is the fecal–oral route. Human volunteers given orally inoculum containing HAstV developed a diarrheic disease with fecal shedding of virus or/and production of specific antibodies to the virus (74, 101). Experimentally, poults inoculated orally with astrovirus produced infection (132, 145). Furthermore, person-to person (in human beings) and contact (in animals) spread have also been observed (120). For instance, caregivers of infected children, including parents, teachers, and medical personnel, did develop illness (2, 70, 72, 118, 119). An outbreak of acute gastroenteritis associated with astrovirus infection among military recruits was also documented (11). Naïve poults developed illness when exposed to infected litter or infected birds (131). In addition to feces/gut contents, infectious astroviruses was detected in water, sewage, sludge biosolids, and naturally grown oysters (22, 23, 60, 124, 125).
1.8 Treatment, Prevention, and Control

The role of astroviruses as pathogens in human beings or animals has not been well defined. In particular, there is little data about the interaction between virus and hosts as well as the immunity of astrovirus. Moreover, patients or animals with diarrhea caused by astroviruses usually shed large numbers of viruses (72), and the astroviruses are extremely stable in the environment, especially in feces, and resistant to inactivation by commonly used disinfectants (1, 75, 139, 164). Consequently, there are no efficaciously therapeutic or prophylactic measures for the prevention or control of astrovirus infections. There are no vaccines available either. Thus, interruption of virus transmission is the key measure that can be instituted currently to prevent astrovirus infection. For human astrovirus infection prevention, universal hygienic procedures must be enforced in such setting, as hospitals, day care center, and families, where person-to-person transmission may occur (95). The treatment of gastroenteritis caused by astrovirus in humans is not as important as prevention since this disease is generally a mild, self-limiting illness. If necessary, i.e., in severe conditions, the conservative measures to treat diarrhea can be administrated. For turkey astrovirus infection prevention and control, strict biosecurity and good management practices emphasizing cleaning, disinfection, litter management, and all-in-all-out operation are the most important measure (131). It is better to depopulate once the flock becomes affected, and replace with agent-free birds. Even though, one study demonstrated that some farms continued to be problematic. To find more effective disinfectants used in the field conditions are urgent. It is important to develop accurate diagnostic tools, investigate the virus epizootiology and pathogenicity,
antigencity, immunity, and molecular characterization to make good prevention and control strategies possible.

REFERENCES


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CHAPTER 2

PHATHOGENICITY OF TURKEY ASTROVIRUS INFECTION IN TRUKEY EMBRYOS AND POULTS

2.1 SUMMARY

A pathogenicity investigation was carried out in specific pathogen free (SPF) turkey embryos and commercial pouls. Turkey astrovirus 1987 (TAstV1987) isolate and turkey astrovirus 2001 (TAstV2001) isolate were used in this investigation. Both virus isolates caused gastroenteritis and growth depression in SPF turkey embryos and pouls. The histopathologic lesions in thymus and bursa induced by TAstV2001 in embryos and pouls were not remarkable. Similarly, no abnormal morphologic changes occurred with thymus of embryos and pouls inoculated with TAstV1987. There was no statistically significant difference in bursa weight / body weight ratios (P>0.05). However, TAstV1987 infection resulted in microscopic lesions of infected bursas of embryos and pouls. These findings indicated that both TAstV1987 and TAstV2001 are important etiologic agents of turkey gastroenteritis. TAstV1987 might cause impairment of the
immune system of the infected poults. The pathogenicity of TAstV1987 is somewhat different from TAstV2001.

### 2.2 INTRODUCTION

Astrovirus was first described by Appleton and Higgins (1) in 1975. They observed small round virus particles of 29 to 30 nm in diameter in the stools of infants hospitalized with mild diarrhea, and vomiting. In the same year, Madeley & Cosgrove (11) observed small round viruses with a five- or six-pointed star-like appearance by direct electron microscopy (EM) in infantile viral gastroenteritis. They coined the name astrovirus based on the distinctive star-like-shaped surface features of these viruses. Afterwards, astroviruses were identified in association with gastroenteritis in a wide range of species, such as lambs (24), calves (29), piglets (3), dogs (12), turkey poults (13, 17, 20), red deer (27), cats (6), mice (8), and minks (4). In addition to causing intestinal illness, extraintestinal illness was also noted in ducklings and chicks (5, 7).

Turkey astrovirus (TAstV) was first detected by McNulty et al in UK (1980) (13) in the feces of poults experiencing diarrhea and increased mortality. Subsequently, Saif, et al. (1985), and Reynolds et al. (1986), identified this virus in the US (17, 18, 20, 21). Later, astroviruses were shown to be widely distributed in commercial turkey poults. In one survey, Reynolds et al. (19) found astroviruses in 78% of diseased turkey flocks with diarrhea, being the most frequently detected enteric viruses. In the eighties, all detected astroviruses were strictly confined to the gastrointestinal (GI) tract of turkey poults, and there was no evidence that astroviruses could cause a systemic disease. The co-infection
of astrovirus and group D rotavirus is the most frequently observed in naturally affected poult (18). In the nineties, TAstV was found not only in the intestines, but also in thymus, and bursa, suggesting a possible effect on the immune system. TAstVs have been incriminated as one of the etiologic agents of turkey poult enteritis and mortality syndrome (PEMS), a devastating multi-system disease (15, 22, 30, 31). PEMS is usually caused by multiple-agents. Studies over the past two decades have primarily focused on identifying causative agents or combined infections, whereas studies on the role of each individual enteric virus in PEMS were limited (2, 9, 10, 13, 15, 17, 21, 22, 25, 26, 31). There are few studies on the pathogenicity of TAstV infection alone (15, 17, 25, 26). TAstVs can result in not only intestinal, but also lymphoid organ infections (15, 17, 25, 26). The gross and microscopic lesions of the small intestines of affected poult are well documented (25, 26). There is only one brief communication and case report on histopathology of the thymus, and bursa of infected birds with PEMS (2). Generally, the exact role of astroviruses in PEMS is not completely clear (9, 10, 31). Astroivirus is a single-stranded positive RNA virus. The polymerase of astroivirus has no proof reading function during the course of replication, so this virus is an error-prone and has very high mutation rates. To date, the diversity in pathogenicity was not documented.

In the present study, we have examined and compared the pathogenicity of two turkey astrovirus isolates, namely, TAstV1987, and TAstV2001 in 26 days old SPF turkey embryonated eggs and 14-day –to-18-day-old turkey poult.
2.3 MATERIALS AND METHODS

Embryonated eggs and turkey poult. All eggs were obtained from our specific pathogen free (SPF) turkey flocks maintained at the FAHRP/OARDC, and were incubated at our facilities. The SPF turkey flock is free of turkey pathogens including enteric viruses. Two-wk-old turkey poult. were generously provided by the Animal Sciences Department, OARDC. Before use, all the poult. were tested for presence of astrovirus by reverse transcription and polymerase chain reaction (RT-PCR) (31) and for antibodies by enzyme-linked immunosorbent assay (ELISA). Poults were housed in a disease containment building that has rooms with HEPA-filtered intake and exhaust air. Birds were provided with feed and water ad libitum.

Viruses. TAsV1987 and TAsV2001 used in this study were isolated in our laboratory from the feces of commercial poult. with diarrhea or PEMS. Originally, both TAsV1987 and TAsV2001 were mixed with other large viruses, such as rotavirus and coronovirus. To separate astrovirus, each mixture was filtered serially through 0.8 µm, 0.45 µm, 0.2 µm, 0.1 µm, and 0.05 µm. Subsequently, filtrates were examined by EM or IEM to make sure the astroviruses were successfully purified. The filtrates containing only astrovirus were used as inocula for virus adaptation and propagation. Twenty two-day-old SPF turkey embryonated eggs were first inoculated with 0.2 ml of the filtrate via the amniotic cavity route as described earlier (23). At 4 DPI, the intestines of the infected embryos were harvested, diluted 1:5-10 (w/v) in 0.05 M Tris-HCl buffer containing 0.15
M NaCl and 15 mM CaCl₂, pH 7.5 (TNC), and homogenized. After freezing and thawing three times, the homogenates were clarified by centrifugation at 3,000 x g, 4°C for 30 minutes. The supernatants were filtrated through 0.45 µm syringe filter. The filtrates were then examined by EM, and used as inoculum for subsequent virus propagation in turkey embryos. Both TAstV1987 and TAstV2001 were passed 8 times in embryos. The last filtrate from passage 8 intestinal homogenates of infected embryos was used in this study.

The titrations for both viruses in turkey embryonated eggs were made using the method described by Villegas (28). The titers were expressed as mean embryo infective dose 50 (EID₅₀), calculated by the method of Reed and Muench (16).

**Experimental design.**

**Trial 1.** Forty 22-day-old SPF turkey embryonated eggs were randomly allotted into four groups of 10 eggs each. Group 1 was inoculated with 100 EID₅₀ of TAstV1987 in 0.2 ml; group 2 was inoculated with 0.2 ml of SPF turkey embryo intestine homogenate mock, which was negative control; group 3 was inoculated with 100 EID₅₀ of TAstV2001 in 0.2 ml; and group 4 was treated as group 2, a negative control. After 96 hours incubation, all eggs were chilled at 4°C overnight. The intestine, thymuses, and bursas from the embryos were examined for gross lesions. Thymuses and bursas were collected for histopathologic analysis.

**Trial 2.** Seventy five (pre-tested astrovirus antigen and antibody free) 2-wk-old naïve poults were randomly assigned to five groups. Each group had 15 birds that were tagged and weighed (day 0). The treatments for each group are summarized in Table 2.1.
Clinical signs were monitored every day during the experimental period. At day 2, 3, and 4 PI, 5 pouls from each group were euthanatized. Body weight and bursa weight for each bird was determined, and the intestines, thymuses, and bursas of birds from inoculated and control groups were observed for gross lesions. Thymuses and bursas were collected for histophathologic analysis.

**Histopathology.** Thymuses and bursas from control and infected embryos and birds were fixed in 10% prefer fixative solution (Antech, LTD. MI, 490T5), and processed regularly. Sections of thymus and bursa were stained with hematoxylin and eosin (H&E), examined by the light microscopy with a digital camera equipment (LEICA DMiRB, Leica Microsystems Wetzlar, Germany), and photographs of the sections were taken using the same microscopy.

**Statistics analysis.** The mean body weight (BW) and the average bursa/BW ratios were analyzed by one-way analysis of variance (ANOVA) and the Turkey’s pairwise multiple comparisons (The Student Edition of MINITAB, Release 12, Minitab Inc. State College, PA). The significance level was defined at $P \leq 0.05$.

**2.4 RESULTS**

**Virus isolation and propagation.** Both TAstV1987 and TAstV2001 were successfully propagated and isolated in turkey embryos following inoculation via the
amniotic sac route inoculation. For each virus isolate, the 8th virus passage in embryos was used as inoculum, and titers of $5 \times 10^5 – 5 \times 10^6$ EID$_{50}$/ml were achieved. TAstV1987 (Fig. 2.2 a) and TAstV2001 (Fig. 2.2 b) of each passage were examined by EM and RT-PCR to confirm the virus presence. Both viruses were shown to be morphologically similar with average size of 30 – 32 nm in diameter. Around 10% of TAstV1987 particles exhibited star-like-shaped appearances (Fig. 2.2 a). However TAstV2001 viral particles did not display very distinguishing surface features (Fig. 2.2 b). Turkey embryos inoculated with either TAstV1987 or TAstV2001 were stunted, but no mortality resulted, and showed distinct intestinal gross lesions. The entire GI tract was severely distended. The gizzards were enlarged, filled with yellowish frothy contents; the intestines were very thin, and fragile, containing greenish or yellowish gaseous fluid.

Pathogenicity. Both groups of embryos infected with TAstV1987 and TAstV2001 exhibited typical gross lesions in the intestines as described above. No changes were observed in the thymus or bursa. Histopathologic examination using light microscopy showed no obvious lesions in the thymuses (Fig. 2.3 c) and bursas of embryos inoculated with TAstV2001 compared with those from non-inoculated SPF control (Fig.2.3 a and Fig. 2.3 b). Similarly, no lesions were found in the thymuses from embryos infected with TAstV1987. However, mild lesions were noted in bursas of this group of birds. The characteristic lesions of infected bursas were primarily lymphocytic depletion, and enlarged follicles (Fig. 2.3 d). On low magnification, there was a decrease in the density of lymphocytes in the medullar region. This was more apparent because of the normally lower lymphocyte density in this zone.

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All groups of inoculated poult and their contacts developed severe diarrhea. The onset of clinical signs was at 2 DPI and persisted throughout the experimental period. The diarrhea was characterized by watery to frothy, yellow or brown droppings, anorexia, depressions and mild dehydration. At necropsy, gross lesions were first noted at 2 DPI as well. The ceca were severely dilated and filled with yellow or green foamy contents. The duodenum, jejunum and ileum were also filled with watery gaseous fluid. Lesions of GI tract induced by TAstV1987 and TAstV2001 infection were similar. Body weights were reduced in the infected and contacted groups as compared with the controls (Fig. 2.1). Histopathologically, thymuses (Fig. 2.4 b) from birds inoculated with TAstV1987 and TAstV2001 and their contacts exhibited no changes in comparison with the negative control group (Fig. 2.4 a). The effect of TAstV1987 and TAstV2001 on bursa weight / body weight ratio is summarized in Table 2.1. Although differences of bursa weight to body weight ratios were observed, statistically they were no significant difference (P>0.05) between infected/contacted groups and normal control group. Microscopically, no lesions were noticed in TAstV2001 affected bursas in comparison with the negative control (Fig. 2.4 c). However, TAstV1987 did induce mild lesions in the bursa at 4 DPI (Fig. 2.4 d), including mild lymphocyte depletion from follicle medullar zones with increased prominence in medullary stroma, and increased stroma beneath the epithelial lining with hypercellularity.
2.5 DISCUSSION

In this study we have isolated and propagated TAstV2001. TAstV1987 was isolated by Reynolds et al. (17-19) at our laboratory in 1987, which was the first turkey astrovirus isolate identified in the USA. We experimentally demonstrated that both TAstV1987 and TAstV2001 isolates induced gastroenteritis in SPF turkey embryos and turkey poults. In addition to causing intestinal illness, TAstV1987 also caused bursa, but not thymus, lesions in embryos and poults. In early reports, the poults challenged with TAstVs alone or combined with other enteric viruses, such as turkey coronavirus, exhibited not only diarrhea, weight loss, and gross lesions (2, 14, 15, 17-21, 25, 30), but also lymphoid tissue atrophy including spleen, thymus, and bursa (15). Yu et al. (30) reported that a small round virus (SRV), which was later determined to be a turkey astrovirus, could cause hemorrhage in the infected thymus, and speculated that the SRV might induce dysfunction of the immune system, which would increase the susceptibility to other opportunistic pathogens. The results from our experiments were consistent with those of earlier reports in term of clinical signs and gross lesions in intestines. However, lymphoid organ atrophy was not found in our study either at the macroscopic or microscopic level. No gross or microscopic lesions were detected in the thymuses of poults and embryos infected with either TAstV1987 or TAstV2001. The only microscopic lesions detected were from TAstV1987 infected bursa, which consisted of mild lymphocyte depletion and stroma increase. Our findings in infected bursa and thymus of challenged embryos and poults were similar to those of Behling-Kelly et al. (2). On the contrary, the results of statistical analysis of bursa weight to body weight
ratios in infected poults were not in agreement with the above report. Virus properties, inoculated virus dose, sampling time, and strain of turkeys may contribute to the difference between our observations and the previous report.

In an earlier study, it was shown that turkey astrovirus infection alone resulted in mortality as high as 11% in challenged poults. In this study, no mortality occurred in the poults affected with TAstV1987 or TAstV2001.

The pathogenicity of TAstV1987 is somewhat different from TAstV2001. The former caused both intestinal and extra-intestinal lesions, and the latter only caused intestinal illness.

Morphologically, characteristic astrovirus star-like-shaped appearance was more prominent in the TAstV1987. SDS PAGE results indicated the two viruses had different capsid protein profiles, which may explain the differences in pathogenicity and antigenicity (data not shown).

Several attempts for turkey astrovirus propagations in continuous cell lines were made by Yu et al. (30). Unfortunately none of them was successful. To date, the only laboratory host for astrovirus isolation and propagation is turkey embryo or poults. In our study, We found that SPF turkey embryos are better host for astrovirus propagation than SPF or commercial poults, and higher virus titers can be achieved by using embryos for virus propagation. Inoculation routes, namely york sac or amniotic cavity, do not make much difference in term of virus titer. We have also found that the propagation of astrovirus, via the amniotic sac route, may help separate turkey astrovirus from rotavirus,
which would be a valuable alternative for separating these two viruses since they co-exist with very high frequency in commercial poult (data not shown).

In conclusion, both TAstV1987 and TAstV2001 are important etiologic agents of turkey gastroenteritis and PEMS. TAstV1987 infection induced mild microscopic lesions, suggesting it might cause some impairment of the immune system. There are some differences in pathogenicity between TAstV1987 and TAstV2001.

2.6 ACKNOWLEDGMENT

The author would like to thank Bob Dearth, Todd Root, and Greg Myers for animal support. Thanks also to Hong Tang for laboratory support. Great appreciation goes to Dr. Lucy Ward, Elzabeth Volk for the preparations of histopathologic slides and slide reading. Many thanks to Dr. Burt Bishop for his kind help on statistical analysis.

REFERENCES


Figure 2.1. Average body weights in experimentally treated and control birds on 0 to 4 DPI. A: pouls were inoculated with TAstV1987 on day 0 (2-wk-old). In this trial, the average body weights of all groups of pouls were not significantly different (P > 0.05). B: pouls were inoculated with TAstV2001 on day 0 (2-wk-old). In this trial, asterisk (*) indicates the average significant difference among treated and control group (P ≤ 0.05).
<table>
<thead>
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<th>Treatment</th>
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<tr>
<td></td>
<td>2 DPI</td>
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<tr>
<td>Control</td>
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<tr>
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<td>1.580&lt;sup&gt;a&lt;/sup&gt;</td>
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</table>

**Table 2.1.** Average bursa weight/body weight ratio of poults inoculated with TAstV1987 or TAstV2001.

Mean values from 2-5 birds. Values with the same lowercase superscripts within a column are not significantly different (P>0.05) from control values.

B: ‘87’ is the abbreviation of TAstV1987.
C: ‘01’ is the abbreviation of TAstV2001.

Group Control was not inoculated.

Group inoculated/87 and inoculated/01 were orally inoculated with 0.2 ml of TAstV1987 and TAstV2001 (containing 100 EID<sub>50</sub>), respectively.

Group Contacted/87 and Contact/01 were placed in the same cage with corresponding inoculated group.
Figure 2.2. Turkey astrovirus particles in fecal or intestinal homogenate preparations from poult or 26-day-old SPF turkey embryos inoculated with TAstV1987 or TAstV2001. A. TAstV1987 from fecal preparation, showing a mixture with turkey coronavirus. B. TAstV1987 isolated from A. C. TAstV2001 in fecal preparation. D. TAstV2001 isolated from C.
Figure 2.3. Photomicrograph of turkey embryo lymphoid tissue sections. A. Normal thymus. B. Normal embryo bursa. C. Thymus from an embryo inoculated with TAstV2001, showing no histopathologic lesions. D. Bursa from an embryo inoculated with TAstV1987 with lymphocytic depletion.
Figure. 2.4. Photomicrograph of poult lymphoid tissue sections. A. Normal thymus. B. Thymus infected with TAstV2001, showing no lesions, C. Normal poult bursa. D. Bursa from a poult Infected with TAstV1987 with mild lymphocytic depletion and hypercellularity (indicated by arrowhead).
CHAPTER 3

ANTIGENICITY OF TURKEY ASTROVIRUSES

3.1 SUMMARY

Astrovirus is a single-stranded positive sense RNA virus. This virus can cause gastroenteritis in humans and in a variety of animal species, including turkey poults. Only human astroviruses were well characterized antigenically. In the current study, two turkey astrovirus isolates were used for characterizing their antigenecity. The antigenecity of TAstV1987 and TAstV2001 were compared with each other, and the antigenic relatedness were determined by using cross-neutralization tests in turkey embryos as well as by testing the cross-reactivity of the two isolates by enzyme-linked immunosorbent assay (ELISA). The antigenic relatedness values (R) were calculated using the Archetti and Horsfall formula. The R value of the cross-neutralization tests was 0.56%, indicating the TAstV1987 and TAstV2001 belong to different serogroups; the R value between two viruses as tested by ELISA was 70.7%, suggesting these two viruses share common antigen(s).
3.2 INTRODUCTION

Astroviruses are small round non-enveloped enteric viruses with a size of 30-32 nm in diameter. Astrovirus was first described by Appleton and Higgins (1) in 1975. Astroviruses primarily cause an acute gastroenteritis in children and young animals including cattle, sheep, swine, dogs, cats, deer, mice, minks, and turkeys (4), (16), (22, 48, 52), (28), (35), (44), (45), (58), (59), (61), (8). Turkey astrovirus was first reported in poults associated with diarrhea and increased mortality by McNulty et al in the UK (1980) (35). Subsequently, Saif, et al. (47), and Reynolds et al. (44, 45), identified this virus in the US. In a survey, Reynolds et al. (46) found astroviruses in 78% of diseased turkey flocks, being the most frequently detected enteric virus. Later on astroviruses were shown to be widely distributed in commercial turkey poults, and an important etiologic agent of enteric disease and turkey poult enteritis and mortality syndrome (PEMS), a highly pathogenic multi-system disease (35), (47), (44).

The antigenecity of human astroviruses are well documented. Currently, eight serotypes were identified by various methods. Five of them were differentiated by immunofluorescence, neutralization, and IEM (17, 27, 29). Serotype 6 and 7 were distinguished from serotype 1 to 5 by IEM (7, 30). The eighth serotype was identified by immunofluorescence assay (3). In general, astrovirus serotype 1 is the most frequent type found (10, 20, 26, 27, 30, 38-42, 49, 60), However the predominant serotype may vary depending on geographic area (12). Hyperimmune antisera derived from animals against the human astroviruses serotypes 1 to 7 react in a serotype-specific manner in IEM and immunofluorescence assays (14, 26, 30). In addition, Herrmann J. E., et al. found that
polyclonal antiserum from rabbit neutralized astrovirus serotype-specifically in a plaque assay (14). They also demonstrated a group specific monoclonal antibody (MAb), a non-neutralizing MAb that can recognize all eight serotypes of human astroviruses (15, 20, 30).

Antigenenically, human, lamb, piglet, red deer, kitten, calf, duckling, chick, and turkey pouls astroviruses are unrelated to each other. The astrovirus infection is strictly species specific, and there is no evidence of cross-reactivity between species (4, 13, 29, 58, 62). For example, convalescent sera from red deer infected with deer astrovirus did not recognize lamb astrovirus antigens in infected lamb intestinal tissue and vis versa (56).

Turkey astroviruses have not been serotyped. There are two astroviruses (TAstV-1 and TAstV-2) whose complete genome sequence data are available in GenBank (accession No. Y15936, and accession No. AF206663). These two TAstVs were classified only based on their genomic sequences and putative amino acid sequences (23).

In the current study, TAstv1987 and TAstV2001 were antigenically compared by cross-neutralization tests in turkey embryos, and a cross-reactivity antibody capture ELISA was also used to determine the antigenic relatedness of the two turkey astroviruses. In summary, results presented in this report indicates that TAstV1987 and TAstV2001 belong to the different serotypes. To our knowledge, this is the first report regarding the turkey astrovirus antigenecity.
3.3 MATERIALS AND METHODS

**Embryonated eggs.** All embryonated turkey eggs were generously provided by the Animal Sciences Department of our institute. To make sure these eggs are astrovirus and its antibody free, the poult(s) from the same hens were tested by RT-PCR and ELISA as described in Chapter 1.


**Virus propagation.** TAstV1987 and TAstV2001 were propagated in 22-day-old turkey eggs as described in Chapter 1. After 4 days incubation at 37 C, the intestines were collected, homogenized, and diluted at 1:10 in 0.05 M Tris-HCl buffer containing 0.15 M NaCl and 15 mM CaCl$_2$, pH 7.5 (TNC).

**Virus purification.** The modified Isopycnic Separation with a Self-Generating Gradient ultracentrifugation method (31) combined with differential centrifugation for turkey atrovirus purification was used. Briefly, turkey embryo intestinal homogenates were frozen and thawed three times, then clarified at 3,000 x g for 30 min at 4 C. The supernatants were collected and centrifuged at 112,000 x g (Beckman LE-80 ultracentrifuge, rotor SW 28) for 2 hours at 4 C through 4-ml 50% (W/W) sucrose cushion. Supernatant was removed. The pellet was resuspended in TNC buffer (pH 7.5), and ultrasonicated three times, and 4.5 ml were mixed with 1.89 grams cesium chloride (CsCl), the mixture was loaded into SW 55 Ti tube (Beckman), and run gradient density ultracentrifugation at 150,000 x g for 18 hours at 4 C (Beckman LE-80 ultracentrifuge, rotor SW 55 Ti). After centrifugation, one band was removed, then resuspended in SW 41 Ti tube with TNC buffer, and pelleted at 107,170 x g for 1 hour at 4 C (Beckman LE-
80 ultracentrifuge, rotor SW 41 Ti). The pellets were resuspended in 0.2 ml of sterile distilled water or 0.01 M pH 7.4 phosphate buffered saline (PBS), and stored at –70 C until used.

**Virus purity examination.** To examine the purity of CsCl purified viruses, the direct electron microscopy (EM) technique was used. A 10 ul volume of purified virus solution was negatively stained with an equal volume of 3% phosphotungstic acid (PTA) solution with 0.4% sucrose, pH 7.0. One drop of stained virus solution was placed on a carbon coated 300 mesh Formvar® copper grid. After drying, the grids were examined for the presence of viruses at 80 kV using a Philips 201 transmission electron microscope (Philips, Norelco, Eindhoven, The Netherlands).

**Determination of virus protein concentration.** Bio-Rad Protein Assay kit was used according to the method of manufacturer (Bio-Rad Laboratories, 2000 Alfred Nobel Dr., Hercules, CA). The concentration of the virus proteins was determined by a comparison of sample absorbance at 595 nm with a standard curve of known concentrations of protein by spectrophotometry.

**Titration of viruses.** TAstV1987 and TAstV2001 were titrated in 22-day-old SPF embryonated turkey eggs. The virus titer was determined as a mean embryo infectious dose (EID$_{50}$). The procedure for virus titration was described in Chapter 1.

**Preparation of hyperimmune sera in Guinea pigs.** Hyperimmune antisera used in cross-neutralization and cross-reactivity tests were prepared in guinea
pigs. The purified TAstV1987 and TAstV2001 were inactivated with 0.1\% β-
propiolactone at 37 C for 2 hr. The inactivated TAstV1987 and TAstV2001 were
emulsified with an equal volume of Freund’s complete adjuvant, respectively. Each
guinea pig was initially immunized subcutaneously with adjuvanted inactivated viruses,
containing 300 µg of viral proteins, at 4 sites on the back. The second and third
immunizations with Freund’s incomplete adjuvant were injected at 2 weeks intervals.
The final boost was given 3 weeks later using purified TAstV1987 or TAstV2001 alone.
Ten days after the last inoculation, blood was harvested, and sera were separated, and
inactivated at 56 C for 30 minutes. The inactivated hyperimmune sera were aliquoted
into 0.2 ml volume and stored at - 20 C until used. The titers of those anti-sera were
determined by antibody enzyme-linked immunosorbent assay (AB-ELISA) described
below.

**Cross-reactivity assay using antibody capture ELISA (AB-ELISA).**

Cross-reactivity of TAstV1987 and TAstV2001 was determined by AB-ELISA
using a procedure similar to that described previously (55). A volume of 50 µl/well was
used for all reagents with the exception of the blocking solution (200 µl/well). Purified
TAstV1987 and TAstV2001 were used as the antigens in an antibody capture enzyme-
linked immunosorbent assay (AB-ELISA) to detect homologous and heterologous anti-
TAstVs antibodies in the hyperimmune srae. A checkerboard titration to determine the
concentration of each reagent in AC-ELISA was first performed. The purified antigens
were diluted to 2 µg/ml in a 0.05 M carbonate-bicarbonate coating buffer (pH9.6), coated
onto MaxiSorp NUNC-Immuno\textsuperscript{TM} 96 MicroWell\textsuperscript{TM} Plates (NUNC, 75 Panorama Creek
Dr., Rochester, NY), and incubated overnight at 4°C. The plates were washed twice with wash solution (phosphate-buffered saline with 0.1% Tween-20 --PBS-T). The blocking agent (3% bovine serum albumin [BSA] PBS-T), was added, and the plates were incubated at 37°C for 3 hours. The plates were washed as described above. Guinea pig anti-TAstV1987 and anti-TAstV2001 hyperimmune sera were serially diluted 10-fold in PBS-T. Each dilution was added into duplicate wells that were pre-coated with homologous or heterologous purified antigens. The preinoculation guinea pig serum and dilution buffer served as negative controls. After 1 hour incubation at 37°C, the plates were washed three times in PBS-T. Goat anti-guinea pig IgG (H+L) horseradish peroxidase labeled antibody (Kirkegaard & Perry Laboratories, 2 Cessna Court Gaithersburg, MD) diluted 1:20,000 in 3% BSA PBS-T was added to each well. Following a 1-hour incubation at 37°C, unbound conjugate was removed by washing five times in PBS-T. The substrate 3,3’, 5,5’-Tetramethylbenzidine (TMB) mixed with equal volumes of Peroxidase Solution B (TMB Microwell Peroxidase Substrate System, Kirkegaard & Perry Laboratories, 2 Cessna Court Gaithersburg, MD) was added for color development. Absorbance was read at 405 nm with a spectrophotometer (MAXline™ Microplate reader, Molecular Devices Corporation, Sunnyvale, CA) after 10 min. The cutoff value was determined by adding 3 x standard deviations to the 2.1 x mean absorbance value of all negative controls. Homologous and heterologous ELISA titers of anti-TAstV1987 and TAstV2001 sera were expressed as the reciprocal of the highest positive dilutions (53-55).
**Virus-neutralization (VN) test.**

A modified method of the virus neutralization test described by Eterradossi *et al.* (9) was employed in turkey embryos. Both TAstV1987 and TAstV2001 were serially passed 8 times in embryos. The 8th passage of the viruses was titrated in embryos, and adjusted to 100 EID$_{50}$/0.1 ml, which were used as challenge viruses in cross-neutralization tests. One hundred forty embryos were used in this test. Every 5 eggs were allotted to one group randomly. The experimental design is summarized in Table 3.1. A constant amount of each virus (100 EID$_{50}$/0.1ml) was mixed with the equal volumes of 10-fold dilution of homologous and heterologous guinea pig anti-sera in PBS (0.01 M, pH7.4). The mixtures of viruses and antisera were incubated at room temperature for 1 hour, and 0.2 ml of the mixture was inoculated into each of 22-day-old turkey embryos by the amniotic cavity. For each dilution, five eggs were used. Three groups of controls were set. Group 1 was only injected with 0.2 ml of each hyperimmune serum; group 2 was inoculated with 0.2 ml of mock serum (SPF turkey serum); and group 3 were challenged with 0.2 ml of each100 EID$_{50}$ virus. The eggs were incubated at 37 C for 96 hours, then the eggs were chilled, and the homologous and heterologous protection titrations were estimated by the method of Reed and Muench (43).

**Determination of antigenic relatedness.**

The antigenic relatedness of TAstV1987 and TAstV2001 was expressed by R-value, which is from the following formula created by Archetti and Horsfall (2).
\[ R = \sqrt{r_1 \times r_2} \]

Where ‘\( r_1 \)’ stands for a ratio, which is determined by dividing the heterologous titer obtained with virus 2 by the homologous titer obtained with virus 1;

‘\( r_2 \)’ stands for another ratio, which is determined by dividing the heterologous titer obtained from virus 1 by the homologous titer obtained from virus 2.

\( R \) is a geometric mean of ratio \( r_1 \) and \( r_2 \), which is used to express the antigenic relatedness between two viruses when both antigens and antiseria were used in cross-neutralization tests.

\( R \) values were interpreted by the method of Brooksby (6) for similarity comparison of two viruses. The criteria is as follows:

- \( R \) value of 1 or close to 1 indicates antigenic identity between two tested viruses;
- \( R \) value > 70% means little or no difference between two viruses tested;
- \( R \) value between 33-70% equals a minor subtype difference;
- \( R \) value between 11-32% equals a major subtype difference;
- \( R \) value between 0-10% equals a serotype difference.

### 3.4 RESULTS

Both TAstV1987 and TAstV2001 were successfully adapted and propagated in turkey embryos via amniotic sac inoculation, and purified by gradient density ultracentrifugation. High yield and pure virus preparations were detected by EM (Fig.
3.1 and Fig. 3.2). To optimize the virus purification procedure, several parameters were compared. For differential centrifugation, different combinations of the concentration and volume of sucrose cushions were initially tested, and 4 ml of 50% (W/W) sucrose was found to be the best, which produced a high purity virus preparation although it somewhat lowered virus yield. For the isopycnic technique, starting with a uniform solution of the sample and gradient material, mixing the sample with CsCl directly, was easier, and more effective for virus separation compared to the method described previously by Yu et al. (63).

Conventional cell culture technique was successfully used to propagate human astroviruses (5). Afterwards, virus serotyping was accomplished (14, 17, 25, 27, 29, 41). Several attempts were made by Yu et al. at our laboratory to propagate turkey astroviruses in several primary and continuous cells. Unfortunately none of these attempts was successful. Since we were unable to propagate TAstVs in cell culture, the antigenic relatedness of TAstV1987 and TAstV2001 were determined using in vivo cross-neutralization in turkey embryos. An AB-ELISA was also employed to investigate the antigenic relatedness of these two astrovirus. AB-ELISA and VN results are summarized in Table 3.1.

3.5 DISCUSSION

Both homologous and heterologous ELISA titers of hyperimmune sera against two turkey astroviruses were much higher than that of VN titers. This higher sensitivity
is probably a result of polyclonal anti-sera recognizing not only neutralizing epitope(s) but also multiple non-neutralizing epitopes. The $R$ value of TAstV1987 and TAstV2001 determined by AB-ELISA titers was 70.7% antigenic relatedness between these two astrovirus isolates. The high $R$ value indicated that the TAstV1987 and TAstV2001 viruses share antigenic determinants, and have little difference according to the criteria of Brooksby (6). However, the $R$ value between these two viruses generated by VN titers was only 0.56%. The low $R$ value indicated that the TAstV1987 and TAstV2001 were antigenically different. According to the criteria of Brooksby, these two turkey astroviruses belong to different serotypes. Grandien et al. (11) defined a true serotype, i.e., the homologous/heterologous neutralization titer ratio should be higher than 16. By this criterion, TAstV1987 and TAstV2001 can be considered true serotypes as well. Koopmans et al. (25) found that the neutralizing antibodies against human astroviruses were predominantly serotype specific with the only exception of low-level cross-reactivity between serotype 4 and serotype1, and typing by neutralization tests (NT) was consistent with typing by ELISA and genotyping. However, high levels of nonneutralizing cross-reactive antibodies have been detected by ELISA by Herrmann and Hudson et al. (14, 17). Our results were discrepant from Koopmans’, but concordant with Herrmann and Hudson’s. Based on the results of this study, it is evident that TAstV1987 and TAstV2001 can not be distinguished in AC-ELISA tests since they share common antigens, but can be distinguished by VN tests due to the differences of their VN epitope(s). These results are similar to that of infectious bursa disease viruses (IBDV) (19, 34, 36).
Generally, phenotyping techniques for the detection of antigenic relatedness of viruses are essential and critical. Several of these techniques have been applied for this purpose. Of these techniques, *in vitro* virus-neutralization (VN) test is very common and useful serological test tool, which is usually used for typing unknown field isolates and characterizing related viruses (18); *In vivo* cross-protection studies are a more important technique since they can not only determine the antigenic relatedness but also evaluate the immunogenecity of related viruses. The latter is specifically valuable to monitor the host response to virus(es). The immunoflorescence, immunosorbent EM, and immune EM methods were originally used to classify the reference types of human astroviruses. Immunogold staining electron microscopy has been used for serotyping astroviruses as well (21). Enzyme immunoassay (EIA), and typing enzyme immunoassay (TYPE-EIA), including the antigen capture ELISA (AC-ELISA) with neutralizing polyclonal antibodies or MAb(s) have been developed to differentiate astrovirus serotypes (25, 37, 41). A simple latex agglutination test for the detection of astrovirus serotype 1, the most prevalent astrovirus, was also developed by Kohno H. *et al.* (24). However the more frequent techniques currently used for the typing of astroviruses are genotyping methods. Reverse transcription-polymerase chain reaction (RT-PCR) with serotype-specific primers were employed to serotype the astrovirus isolates (32, 50, 51, 57, 64). RT-PCR followed by sequencing was also conducted (32, 64). Several reports pointed out genotyping results were fully consistent with that of ELISA (25, 41). A novel heteroduplex mobility assay (HMA) may also be utilized to serotype astroviruses without sequencing RT-PCR amplicons (33).
To conclude, TAstV1987 and TAstV2001 possess common antigen(s), but induce serotype-specific neutralizing antibodies, respectively, and they belong to different serotypes.

3.6 ACKNOWLEDGMENT

The author would like to thank Bob R. Dearth, Todd Root, and Greg Myers for animal support.

REFERENCES


80
Antigenic relatedness of TAstV1987 and TAstV2001

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Table 3.1. VN titers and AB-ELISA titers and antigenic relatedness values (R) of TAstV1987 and TAstV2001.

- **a.** R (relatedness) values were calculated according to Archetti and Horsfall formula.
- **b.** 100 EID<sub>50</sub> of virus dose was used for each individual virus.
- **c.** VN titer was determined by the method of Reed and Muench.
- **d.** The AB-ELISA titer was the reciprocal of the highest serum dilution that was positive.
- **e.** Homologous R value is considered 100%.
Figure 3.1. TAstV1987 particles purified by sucrose and CsCl differential and isopycnic ultracentrifugation. Bar = 100 nm. Particle showing star-shaped surface feature is arrowed.
**Figure 3.2.** TAstV2001 particles purified by sucrose and CsCl differential and isopycnic ultracentrifugation. Bar = 100 nm.
CHAPTER 4
DETECTION OF TURKEY ASTROVIRUSES

4.1 SUMMARY

Turkey astrovirus is an important agent of poult enteritis and mortality syndrome (PEMS). PEMS is an infectious, transmissible disease affecting young turkeys between 1-4 weeks of age. The disease is characterized by diarrhea, anorexia, growth depression, mortality and immunosuppression, and it has caused significant losses to turkey producers in the U. S. A. The diagnosis of astroviruses has been mainly dependent on electron microscopy (EM) or immune electron microscopy (IEM) techniques. To develop other diagnostic assays, an antigen capture enzyme-linked immunosorbent assay (AC-ELISA) was developed using polyclonal hyperimmune antisera against TAstV1987 and TAstV2001 isolates, and monoclonal antibody (MAb) for TAstV2001. Monoplex and multiplex reverse transcription polymerase chain reactions (RT-PCR) were developed as well using non-degenerate primer sets specific to the capsid region, and degenerate primer pairs covering the polymerase area of TAstV genome. One ssRNA internal control (IC) template reagent was produced, and applied to the RT-PCR to reduce the false negative rate of the test. Direct EM was used also in this study and the
results obtained from both EM and RT-PCR were compared. The AC-ELISA with polyclonal antisera had higher sensitivity and wider detection spectrum than that of the monoclonal AC-ELISA with group-specific MAb, whereas the monoclonal AC-ELISA had very high specificity but lower sensitivity. The sensitivity of monoclonal AC-ELISA is 0.06 µg of viral proteins. Fecal samples or gut content samples from naturally infected or experimentally infected poults with PEMS were examined by EM and RT-PCR. The EM results indicated that the positive rate of small round viruses (SRVs) that could include astroviruses as well as enteroviruses was 33.4%. The monoplex RT-PCR results amplified with primers SRV-1-3 and SRV-1-5 revealed that the positive rate of astroviruses was 45.3%, which is 10.9% higher than that of direct EM even if other SRVs were not excluded. Multiplex RT-PCR with both SRV-1-3 and SRV-1-5 and AFCP-F1 and AFCP-R1 and the monoplex RT-PCR with degenerate primers showed good specificity and wider detection spectrum than that of earlier published data and the monoplex RT-PCR with the positive rate of 59.4%, 25% higher than that of EM. With the internal control, an overall test inhibition rate of 12.5% was found for the astrovirus RT-PCR assay, which can be used to decrease the false-negative rate of the detection.
Astroviruses are etiologic agents of acute gastroenteritis in the young of a variety of species, including humans, cattle, sheep, swine, dogs, cats, deer, mice, minks, and turkeys (3), (17), (46), (27), (32), (37), (38), (48), (52), (53), (7). In addition to causing intestinal illness, extraintestinal illness was also noted in some avian species (9, 18). Human astroviruses were first described in 1975 by Appleton and Higgins (1), Madeley and Cosgrove (29). Turkey astrovirus was described by McNulty, M. et al. (32) (1980), Saif, L. J. et al. (41) (1985) and Reynolds, D. L. et al. (37)(1986). Astroviruses are small round, non-enveloped viruses with size of 28 to 30 nm in diameter. The remarkable feature of astroviruses is that about 10% of the particles have a five or six-pointed star-like surface structure when examined by direct electron microscopy (EM). Turkey astroviruses (TAstV) have been incriminated as an etiologic agent of enteric disease and turkey poult enteritis and mortality syndrome (PEMS), a highly pathogenic multi-system disease (32), (41), (37). TAstV infections are geographically widespread (36). In a survey of commercial turkey flocks with enteric disease using EM and electropherotyping, astroviruses were the most commonly detected viruses (39). Previously, detection and diagnosis of astroviruses has been heavily dependent on EM, IEM, and fluorescent antibody assays. However, the typical distinctive star-like-shaped morphology of astroviruses is pH dependent, and can vary among different preparations (4, 31). Yu and coworkers (55) reported that the turkey astrovirus they detected was featureless. Therefore, it is difficult sometimes to differentiate astroviruses from other small round viruses, such as enteroviruses, picornaviruses, etc. by EM or IEM based only
on their capsid profiles. Furthermore, the sensitivity of EM and IEM is relatively poor. The fluorescent antibody assays are not practical for testing a large number of samples, and its sensitivity and specificity is also not very high. An enzyme immunoassay (EIA) for detection of human astrovirus was developed (15). Cubitt et al. demonstrated that both EM and EIA to screen for the presence of astroviruses in stools had a similar level of sensitivity but EIA was more practical for monitoring an extensive outbreak. Recently, the polymerase chain reaction (PCR) for human astrovirus has been shown to be a useful diagnostic technique with higher sensitivity than that of EM and IEA (19). For turkey astrovirus detection, Yu et al. (55), and Koci et al. (23) developed reverse transcription-polymerase chain reaction (RT-PCR) methods. The detection spectrum of both RT-PCR methods is not broad since all the primer pairs used in the RT-PCR reactions were designed from only one turkey astrovirus (TAstV) sequence data.

Our objective in this study was to develop several highly sensitive and specific diagnostic tests for detection of turkey astroviruses. For this reason, two AC-ELISAs were established using polyclonal hyperimmune antisera against TAStV1987 and TAStV2001 isolates, and monoclonal antibody (MAb) for TAstV2001; and genotyping methods, monoplex and multiplex reverse transcription-polymerase chain reaction (RT-PCR) with a wide detection spectrum, were established as well. One ssRNA internal control (IC) template reagent was developed, and applied to the RT-PCR reactions to monitor amplification and detection, and to lower the false negative rate of the test by retesting inhibitory specimens. In addition, direct EM was used, and the results obtained from both EM and RT-PCR were compared.
**4.3 MATERIALS AND METHODS**

**Fecal or gut content specimens.** Three hundred and nineteen field fecal samples from turkey poultts affected with PEMS from North Carolina commercial turkey farms were collected during May to August, 2001. These specimens were treated as described previously (55). Briefly, samples were mixed well with 0.01 M pH 7.4 phosphate buffered saline (PBS) buffer at a ratio of 1:10, and frozen and thawed three times. The samples were then clarified by centrifugation at 3,000 x g for 30 min at 4 C. Supernatants were collected and filtered subsequently through 0.8 µm and 0.45 µm filters. Filtrates were then stored at –70 C until examined.

**Viruses.** TAstV1987 and TAstV2001 were used for production of hyperimmune antisera which severed as capture antibody and detective antibody as well as the positive controls in polyclonal AC-ELISA assay. TAstV2001 was employed as antigen to produce monoclonal antibodies, which were used as detective antibody in the monoclonal AC-ELISA. TAstV1987, TAstV1997 (SRV p14), and TAstV2001 were utilized as positive controls in the monoclonal AC-ELISA assay and all RT-PCR reactions. Turkey group A rotavirus (TgARV), turkey group D rotavirus (TgDV), turkey atypical rotavirus 202 JP1 ATR, turkey coronavirus (TCV, Minisota strain), turkey enterovirus (TEV), and human group A rotavirus (Wa-Rota) were used to determine the specificity of the monoclonal AC-ELISA. Purified TAstV2001 with CsCl was used to determine the sensitivity of the monoclonal AC-ELISA.
**Virus propagation.** The procedure used for the propagation of TAstV1987 and TAstV2001 was described in chapter 1. Twenty two-day-old specific pathogen free (SPF) turkey embryos were inoculated each with a virus preparation containing $10^5$ EID$_{50}$/0.2 ml of viruses via the amniotic sac. After 4 days incubation at 37 C, the intestines were collected, homogenized, and diluted at 1:10 in 0.05 M Tris-HCl buffer containing 0.15 M NaCl and 15 mM CaCl$_2$, pH 7.5 (TNC).

**Virus purification.** The differential centrifugation and a modified isopycnic separation with a Self-Generating Gradient ultracentrifugation method (30) for turkey atrovirus purification were used as described the chapter 1. Briefly, turkey embryo intestinal homogenates were frozen and thawed three times, then clarified at 3,000 x g for 30 min at 4 C. The supernatants were collected and centrifuged at 112,000 x g (Beckman LE-80 ultracentrifuge, rotor SW 28) for 2 hours at 4 C through 4-ml 50% (W/W) sucrose cushion, and the supernatant was removed. The pellet was resuspended in TNC buffer (pH 7.5), and ultrasonicated three times on ice, 30 seconds each time. The resuspension (4.5 ml) was mixed with 1.89 grams cesium chloride (CsCl), and the mixture was loaded into SW 55 Ti tube (Beckman), and separated by gradient density ultracentrifugation at 150,000 x g for 18 hours at 4 C (Beckman LE-80 ultracentrifuge, rotor SW 55 Ti). After centrifugation, one band was removed, then resuspended in SW 41 Ti tube with TNC buffer, and pelleted at 107,170 x g for 1 hour at 4 C (Beckman LE-80 ultracentrifuge, rotor SW 41 Ti). The pellets were resuspended in 0.2 ml of sterile distilled water or PBS, and stored at –70 C until used.

**Determination of virus protein concentration.** The protocol for measuring the virus protein concentration was described in chapter 3.
**Polyclonal AC-ELISA**

**Antisera.** The first antibodies (capture antibody) used in the polyclonal AC-ELISA were prepared by inoculating turkey poult or chickens with purified TAstV1987 and TAstV2001. For turkey antiserum production, the procedure was a modification of that described by Hayhow *et al.* (13). Briefly, 2-wk-old SPF turkeys were initially inoculated orally with $10^4$ EID$_{50}$/0.2 ml TAstV1987 and TAstV2001, respectively. Then the birds were injected 3 to 4 times subcutaneously at bi-weekly intervals with adjuvantated inactivated viruses purified with CsCl. The inactivated TAstV1987 and TAstV2001 were emulsified with an equal volume of Freund’s complete adjuvant, respectively. After two wks, each of the previously inoculated turkey poults was injected subcutaneously with above emulsion of viruses, containing 300 µg of viral proteins in the neck region. The second and third immunizations with Freund’s incomplete adjuvant were within 2 weeks intervals. The final boost was given after 3 weeks using purified TAstV1987 or TAstV2001 alone. Ten days after the last inoculation, blood was harvested, and sera were separated, and inactivated at 56°C for 30 minutes. The inactivated hyperimmune sera were divided into 0.2 ml aliquots and store at -20°C until used. The titers of the anti-sera were determined by antibody enzyme-linked immunosorbent assay (AB-ELISA) described previously (in chapter 3).

The procedure for the production of chicken anti-TAstV1987 and TAstV2001 was similar to that of turkey anti- TAstV1987 and TAstV2001 except that the first inoculation in chickens was given by injection.
The second antibodies (detective antibody), anti-TAstV1987 or TAstV2001 were prepared in guinea pigs as described in chapter 3. To remove any possible anti-intestinal tissue antibodies, the second antibodies were absorbed with freeze dried SPF turkey intestinal tissue powder before use.

**Polyclonal AC-ELISA.** The procedure used for the polyclonal AC-ELISA was performed as described earlier with modification (6, 13). A standard volume of 50 µl/well was used for all reagents with the exception of the blocking solution (200 µl/well). A checkerboard titration to determine the concentration of each reagent in AC-ELISA was first performed. The first antibody, turkey anti-TAstV2001 or TAstV1987, diluted 1:800 in a 0.05 M carbonate-bicarbonate coating buffer (pH9.6) was coated onto MaxiSorp NUNC-Immuno™ 96 MicroWell™ Plates (NUNC, 75 Panorama Creek Dr., Rochester, NY), and then incubated overnight at 4 C. The plates were washed twice with wash solution (phosphate-buffered saline with 0.1% Tween-20 --PBS-T). The blocking agent, (3% bovine serum album [BSA] PBS-T), was added, and the plates were incubated at 37 C for 3 hours. The plates were washed as described above. Fecal specimens diluted 1:5 in PBS-T were added into the coated wells in duplicate and incubated for 1 hour at 37 C. The plates were rinsed three times with washing buffer. Guinea pig anti-TAstV1987 or anti-TAstV2001 hyperimmune sera diluted 1:12,800 in PBS-T were then transferred into the wells, and incubated for 1 hour at 37 C. After washing 3 times in PBS-T, goat anti-guinea pig IgG (H+L) horseradish peroxidase labeled antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD) diluted 1:20,000 in 3% BSA PBS-T was added to each well. Following a one-hour incubation at 37 C, the unbound
conjugate was removed by washing five times in PBS-T. The substrate 3,3’, 5,5’-Tetramethylbenzidine (TMB) mixed with equal volumes of Peroxidase Solution B (TMB Microwell Peroxidase Substrate System, Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added for color development. Absorbance was read after 10 min at 405 nm with a spectrophotometer (MAXline™ Microplate reader, Molecular Devices Corporation, Sunnyvale, CA). The cutoff value was determined by adding 3 x standard deviations to the 2.1 x mean absorbance value of all negative controls.

**Monoclonal AC-ELISA.**

Production of hybridoma secreting monoclonal antibodies against TAstV2001. The procedure was a modification of that described previously (24). Six-wk-old female BALB/c mice were initially inoculated intraperitoneally (ip) with 100 µg of CsCl purified TAstV2001 emulsified with an equal amount of Freund’s complete adjuvant. After two wks, the mice were immunized 3 times ip at bi-weekly intervals each time with 50 µg of purified virus mixed with equal volume of Freund’s incomplete adjuvant. Three days before fusion, the last boost was given ip with 50 µg of purified TAstV2001 alone. Hybridomas were prepared by fusing splenocytes from these immunized mice with SP2/0 myeloma cells at a ratio of 4:1 using polyethylene glycol 1500 (Sigma, St. Louis, MO). Hybrid cells were seeded onto plates in which feeder cells, peritoneal macrophages from BALB/c mice, were pre-plated in enriched RPMI-1640 with hypoxanthine-aminopterin-thymidine (HAT) (GIBCO™ Invitrogen Corp. Grand Island, NY) at the day before fusion. On day five of incubation, all wells were filled with HAT medium. After three
days incubation, half of the medium was removed from each well, and replaced with hypoxanthine-thymidine (HT) medium. Hybridomas secreting MAbs specific to TAstV2001 were screened by antibody ELISA similar to that described in chapter 3. Positive clones were subcloned 2 to 3 times using the limiting dilution technique (11). Cell culture supernatants from positive hybridomas were collected, aliquoted, and store in –20 C until used. The resulting positive hybridomas were then stored in liquid nitrogen.

**Hybridoma characterization and application.**

**Determination of isotype and subisotype.** ELISA tests were used for isotyping hybridoma supernatant fluids. A commercial mouse monoclonal antibody isotyping reagent, including monospecific antiserum to mouse IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA, was obtained from Sigma (St. Louis, MO). The tests were performed following the instructions of the manufacturer. Briefly, each isotype or subtype monospecific antiserum, diluted 1:1,000 in a 0.05 M carbonate-bicarbonate coating buffer (pH9.6) was coated onto MaxiSorp NUNC-Immuno™ 96 MicroWell™ Plates (NUNC, 75 Panorama Creek Dr., Rochester, NY), and then incubated overnight at 4 C. The plates were washed twice with wash solution (phosphate-buffered saline with 0.1% Tween-20–PBS-T). The blocking agent, (3% bovine serum albumin [BSA] PBS-T), was added, and the plates were incubated at 37 C for 3 hours, and then washed as described above. Individual cell supernatant fluid from positive hybridomas was added into the well in duplicate and incubated for 1 hour at 37 C. The rest of the steps were similar to those described in AB-ELISA in chapter 3.
**Virus-specific reactivity pre-tests.** To pre-determine the reactivity of each MAb, Three antibody capture ELISA tests using purified TAstV1987, TAstV1997, and TAstV2001 as coating proteins were developed as described in chapter 3.

**Monoclonal AC-ELISA.** Monoclonal AC-ELISA was similar to that of polyclonal AC-ELISA described above. In this test, turkey anti-TAstV2001 or anti-TAstV1987 hyperimmune antisera were used as the capture antibody. Two group-specific MAbs, 1-8E and 1-9D were employed as detective antibodies, and goat anti-mouse IgG (H+L) horseradish peroxidase labeled antibody was used as conjugate. Of 319 field fecal samples, 116 were tested by this method. The gut homogenates of 26-day-old turkey embryos inoculated with TAstV1987, TAstV1997 (SRV p14), and TAstV2001 severed as positive control. The intestinal mock from SPF turkey embryos and the feces of SPF turkey poult as well as PBS were used as negative controls. The TAstV2001 purified by CsCl was used to determine the sensitivity of the test, and other enteric viruses, including turkey enterovirus, turkey coronavirus, human rotavirus, turkey rotavirus, were tested to verify the specificity of this AC-ELISA.

**Reverse transcription-polymerase chain reaction (RT-PCR) for detection of astroviruses in fecal specimens.**

**RNA extraction.** The RNA was extracted from filtrates of fecal specimens using a commercial kit (TRIZOL LS Reagent, Life Technologies, Grand Island, NY) following the manufacturer’s instructions. Briefly, 0.25 ml of the filtrates was mixed with 0.75 ml
of TRIzol LS Reagent. After 10 minutes incubation at room temperature, 0.2 ml of chloroform was added for phase separation. The mixture was centrifuged at 12,000 x g for 15 minutes at 4°C and the aqueous phase was collected. Isopropyl alcohol was added to the aqueous phase to precipitate the RNA. After centrifugation at 12,000 x g for 10 minutes at 4°C, the pelleted RNA was washed with 75% Ethanol (prepared in DEPC-treated H₂O), dried for 30 minutes at room temperature, and dissolved in 22 µl of DEPC-Treated H₂O at 56°C for 10 minutes. The RNA concentration was determined spectrophotometrically by measuring the A260/280 ratio (Maniatis, T. et al., 1982). RNA was stored at –20°C until used.

**Oligonucleotide Primers.** Three sets of oligonucleotide primer pairs were used in the RT-PCR (Table 4.1). Information on the first set of primer pairs was kindly provided by Dr. Akbar Ali and Dr. Donald L. Reynolds, Iowa State University. They were referred to as SRV-1-3 and SRV-1-5 with an expected product of 473 nucleotides. The second set of primers was designed based on the sequence data of TAstV-2 (accession number AF206663), referred to as AFCP-F1 and AFCP-R1 with an expected product of 464 nucleotides. Both above primer sets are specific to the 3’-end capsid region of the turkey astrovirus RNA genome. The third primer pairs were degenerate primers specific for a conserved sequence covering a 601 nucleotide region based on the polymerase region of two complete turkey astrovirus sequences in GenBank (accession Numbers Y15936 and AF206663). They were designated TAPG-l and TAPG-R1.
All primers were designed by using primer designing software Primer 3 (http://www.yk.rim.or.jp/~aisoai/tool.html), and synthesized by INTEGRATED DNA TECHNOLOGIES, INC. (Coralville, IA).

**Monoplex RT-PCR and multiplex RT-PCR.**

**RT-PCR.** RT-PCR was performed using a Titan One Tube RT-PCR System Kit (Roche Molecular Biochemicals, Mannheim, Germany). For each reaction, 5 µl of the extracted total RNA, 5 µl of DEPC-treated H₂O, 1 µl of DMSO, 1 µl of upstream primer and 1 µl of downstream Primer was added to a 0.2 ml thinwalled PCR tube. The tube was heated at 70 °C for 10 min, then chilled on ice, and tested according to the manufacturer’s recommendations. The master mix 1 and master mix 2 solutions were added up to 50 µl respectively. The reverse transcription was performed at 45 °C for 90 min. The PCR amplification was carried out with GeneAmp PCR System 9700 (Perkin-Elmer, Foster City, CA). The cycling parameters for each reaction were 1 cycle at 94 °C for 3 minutes, followed by 35 cycles of: 94 °C for 1 min., 42 °C for 2 min, and 72 °C for 1 min 30 sec., with a final elongation step for 7 minutes at 72 °C. After completion of the PCR, 10 µl of the PCR product was loaded onto a 1.5 % agarose gel. The DNA bands were separated by electrophoresis, stained with ethidium bromide, and visualized with an UV transilluminator as described previously (Yu. et al., 2000). The PCR product was confirmed by sequencing. DNA from RT-PCR was purified using a QIAquick® kit (QIAGEN Inc., Valencia, CA), then the nucleotide sequence was determined using an ABI 377 automated DNA sequencer (Perkin Elmer inc.). The forward and reverse
primers were used as sequencing primers in separate reaction tubes. The sequences generated were analyzed using the GCG Version 10 and the online BLAST search for sequence similarity program (http://www.ncbi.nlm.nih.gov/BLAST) to search for similarity to sequences published in the GenBank.

**Electron microscopy (EM).** Direct EM was performed as described previously (55).

**The production of a ssRNA internal control (IC) template.** The general procedure was a modification of Wang’s protocols (49), and illustrated in Fig. 4.1.

**Internal primer set design.** Previous RT-PCR results indicated that the SRV primer set can only amplify TAsT2001, but not TAsT1987 or TAsT1997, and the AFCP primer set can only be used to amplify TAsT1987 and TAsT1997, but not TAsT2001. To increase the detection spectrum, i.e., internal control template to serve as the positive indicator in a multiplex RT-PCR system, both primer sets sequence data, as well as pCR 2.1 vector sequence, were utilized to design the internal control primer. Int-L1, the upstream primer, consists of SRV-1-5, AFCP-F1, and 20 nt of pCR 2.1 vector sequence (position 1007 – 1016) in order from 5’ to 3’ end. The sequence of this primer is TCT ATT GGG ATA CAA CAG GCA GGC GGC ACA AGG TGA CAA CAG GAT ATA CAC AGG AAG CGG AAC ACG TAG AA; Int-R1, the downstream primer, starts with AFCP-R1 at it’s 5’ end followed by SRV-1-3 and 20 nt of pCR 2.1 vector sequence (position 1487-1506) at its 3’ end with the sequence of ATC CTT GAT ATG CTG TTT GAG AGT CAG GAA TGA CCA CTG GCA GAA GAT TGT CGT CCT GCA GTT CAT TCA
G. The internal control primer pairs were analyzed for EcoRI and HindIII restriction sites free using online software program (http://www.firstmarket.com/cgi-bin/cutter) since these two restriction enzymes would be used late in the production of the IC. The primers were synthesized and purified by INTEGRATED DNA TECHNOLOGIES, INC. (Coralville, IA).

**Synthesis of 601 bp IC insert.** For generation of a 601 bp IC insert, the linearized pCR 2.1 vector (Invitrogen, Carlsbad, CA) used as a template was amplified by PCR with internal primer set, Int-L1 and Int-R1. The PCR reaction was performed in a volume of 50 µl, in which 1 ng (10ng/ µl) of pCR 2.1 vector template was added into 49 µl of the reaction mixture containing 5 µl of 10 x PCR buffer (Promega, Madison, WI), 2.5 mM MgCl₂, 0.5 mM of each dNTP, 0.5 mM of each primer, and 2.5 U Taq DNA polymerase. Forty cycles of denaturation at 94 C for 30 s, annealing at 54 C for 60 s, and elongation at 72 C for 90 s were run in a GeneAmp PCR System 9700 (Perkin-Elmer, Foster City, CA). The amplicon was separated by electrophoresis in 1.5% agarose gels, stained with ethidium bromide, and visualized with an UV transilluminator as described previously (55). The DNA from the PCR was purified using a QIAquick® kit (QIAGEN Inc., Valencia, CA),

**Cloning into the TOPO TA vector.** The purified PCR product was cloned into the TOPO TA vector (pCR 2.1, Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. Eight transformed colonies were subcultured in Luria-Bertani (LB) broth medium with 100 µg/ml of carbenicillin (CBC, Life Technology) and grown overnight. The recombinant plasmids were prepared by using QIAprep Miniprep kit (QIAGEN Inc., Valencia, CA) according to the manufacturers’ protocols. Restriction enzyme digestion
(EcoR1) analysis of the recombinant plasmid was performed to confirm the presence of the IC insert, and correct orientation of the insert was determined by sequencing the recombinant plasmid with M13 reverse primer and M13 forward primer.

**In vitro transcription.** To prepare plasmids for the production of “run-off” transcripts, plasmids containing the IC insert were first linearized by *HindIII* (Promega, Madison WI) at the 3’-end. After the restriction digestion, the linearized plasmid was extracted with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with ethanol, and resuspended in nuclease-free water. Then the purified plasmids served as templates in a transcription reaction *in vitro* with a Riboprobe System – T7 kit (Promega, Madison WI) using a modified method of the manufacturer’s procedure. Following transcription, the dsDNA templates were removed by digestion with RNase-Free RQ1 DNase at 37°C for 15 min. The transcripts were purified by phenol:chloroform:isoamyl alcohol (25:24:1, pH 4.5) extraction, 7.5 M ammonium acetate / ethanol precipitation, and washing once with 70% ethanol. The precipitated RNA pellets were dried under vacuum and resuspended in 20 µl of nuclease-free water, and stored at –70°C until used. The concentration of purified RNA template was determined by spectrophotometer at 260 nm.

**Characterization and determination of detecting limit of the IC.**

**Verification of the integrity of RNA IC template.** To verify the integrity of the internal control template, direct agarose gel electrophoresis and RT-PCR tests were employed. The expected size of the RT-PCR product of the IC template should be 749 bp in length, and amplicons from this template in RT-PCR were expected to be 576 bp with the SRV primer set, and 575 bp with the AFCP primer set. Two RT-PCR reactions
were performed with both SRV and AFCP primer sets. The parameters of RT-PCR were followed as described above except for adding only 0.1 µl volume of the purified internal control template RNA.

**Determination of detecting limit of the IC.** A modified procedure from Smiley et al. (45) was used. Ten-fold serial dilutions (10^{-2} – 10^{9}) of the IC reagent were used as templates in individual RT-PCR reactions with the SRV primer set and the AFCP primer set, respectively.

**Application of the IC to the RT-PCR.** Sixty-four fecal samples from turkey poults affected with PEMS from commercial turkey farms were examined using multiplex RT-PCR with primer sets SRV and AFCP. Total RNA extracted from samples with TRIzol reagent (Life Technology) was coamplified with 180 femtogram (1 µl of the 10^{-6} dilution) of the ssRNA internal control, per test. The RT-PCR conditions were described as above, and the specific band profiles amplified were compared.

**4.4 RESULTS**

**Antisera.** All the antisera prepared in guinea pigs, chickens, and turkeys were titrated with AB-ELISA (Table 4.2). The titers of these antisera ranged from 12,800 to 409,600. All antisera prepared against TAstV1987, TAstV1997 (stored at our lab) and TAstV2001, aggregated homogenously and heterologously TAstV1987, TAstV1997, and TAstV2001
when examined by IEM, whereas sera from normal guinea pigs or SPF chickens/turkeys did not (data not shown).

**Polyclonal AC-ELISA.** A total of 64 fecal specimens were tested by polyclonal AC-ELISA. Of these samples, 51 (79.6%) were astrovirus positive. A comparison between EM results and polyclonal AC-ELISA results is illustrated in Table 4.5. The specificity was determined by testing the known positive and negative intestinal samples. All positive and negative samples tested were shown to be positive or negative, respectively. The detective antibody, guinea pig anti-TAstV2001, pre-absorbed with SPF turkey embryo gut homogenates resulted in a low non-specific background. On the contrary, the non-pre-absorbed guinea pig anti-TAstV2001 displayed a high background in the negative control samples. Polyclonal AC-ELISA was more sensitive compared with that of the monoclonal AC-ELISA and RT-PCR results described later.

**Monoclonal AC-ELISA.**

**Production and characterization of hybridoma secreting monoclonal antibodies against TAstV2001.** Twenty five hybridomas secreting specific monoclonal antibodies against TAstV2001 were obtained (Table 4.3). Of these 25 positive hybridomas, 23 secreted group specific monoclonal antibodies, which reacted with TAstV1987, TAstV1997, and TAstV2001; 2 (2-6A and 2-8A) produced strain specific monoclonal antibodies, which only detected homologous virus TAstV2001. All MAbs secreted by the twenty five hybridomas did not react with viruses other than astroviruses. The Ig isotype or subisotype of twenty one out of twenty five MAbs were determined by
an AB-ELISA with a commercial monoclonal antibody isotyping kit containing monospecific antiserum to mouse IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA. The isotypes of MAb 1-5G, 2-10H, and 5-7H were IgG2b, IgG2a, and IgM, respectively, and the rest of them were IgG1. No MAbs belonged to IgG3 and IgA. Among these MAbs, 1-8E and 1-9D had the highest antibody titer, which were used as detective antibody in the monoclonal AC-ELISA.

**Monoclonal AC-ELISA.** One hundred and sixteen of 319 field fecal samples along with both positive and negative control were examined by this test. The positive controls showed a high OD values, and the negative controls had very low OD value (as low as the blank wells), indicating the test was very specific. However, there were only three gut homogenate samples with a virus titer $10^{2.5}$ to $10^{4.5}$ EID$_{50}$ which showed strong positive, and all the rest were negative. The lowest detection limit was determined to be 0.06 µg of viral protein.

**Monoplex RT-PCR in the absence of the internal control template and sequence confirmation.** Fecal specimens or gut contents (N = 319) were examined by monoplex RT-PCR with primer pairs of SRV-1-3 and SRV-1-5. Expected 473 bp RT-PCR products were obtained from 42.2% preparations of these fecal samples of poultts affected with PEMS (Table 4.4, Fig. 4.3). The positive rate was 10% higher as compared with that of direct EM (Table 4.4), indicating the sensitivity of RT-PCR was much higher than that of EM. However, some fecal samples (10/22) were detected only by EM but not by RT-PCR, that is, EM detection was positive but RT-PCR with SRV-1-3 and SRV-15 primers
was negative. This could be due to the wide detection spectrum of the EM as compared to that of RT-PCR since EM detects not only turkey astrovirus but also enterovirus, calicivirus, and other small enteric viruses. False negative caused by inhibitors in the RT-PCR reaction system might be another reason, which would lower the sensitivity of RT-PCR detection as well. The results of RT-PCR with degenerate primers TAPG-L1 and TAPG-R1 showed an expected 601 bp band (Fig. 4.6) with the positive rate of 59.4% of tested samples (N = 64), which is 27.2%, and 17.2% higher as compared with that of EM, and RT-PCR with primer SRV-1-3 and SRV-1-5, respectively. Sequence analysis for several amplicons selected randomly from SRV primer set amplified products showed a high homology with published turkey astrovirus sequence data (GenBank accession # AF206663), confirming that the RT-PCR was specific. It was shown that the SRV primer set could only amplify the TAstV2001, but not TAstV1997 (SRVp14); and a weak amplicon band was visualized for TAstV1987 (Fig. 4.3). The results of monoplex RT-PCR with primer set AFCP-F1 and AFCP-R1 demonstrated that the AFCP could only amplify TAstV1987 and TAstV1997 (SRV p14), but not TAstV2001 (Fig. 4.4).

**Negative staining electron microscopy.** The same samples (N =319) were tested by EM. Partial viral particles detected in preparations from fecal specimens of poults affected with PEMS by EM with negative staining were illustrated in Fig. 4.2. The viral particles C-1 and C-2 are small round viruses (SRVs) including turkey astrovirus. The average size of the particles of SRVs is around 28 +/- 2 nm. The positive rate of SRVs was 32.2% (Table 4.4). In addition to SRVs, other viral particles such as rotavirus, coronavirus (TCV), mini-reovirus, and adenovirus as well as bacterial phages were
detected in some of fecal samples. The frequency of these particles is summarized in Table 4.4. Furthermore Alfalfa mosaic-like virus was observed in 3 specimens out of 319 samples. The majority of these fecal samples were shown to contain multiple viral types.

**Production of a ssRNA internal control (IC) template.**

A 601 bp cDNA, which consisted of the internal primer sequences and a 460 bp flanked fragment of pCR 2.1 (located between position 1016 and 1487), was synthesized by RT-PCR using 1 ng of pCR2.1 vector as template, and constructed into the TOPO TA vector. The positive clones were confirmed by EcoR1 digestion and electrophoresis and sequence analysis of recombinant plasmids. The recombinant plasmids containing forward orientation of the T 7 promoter were selected for production of a sense ssRNA internal control template reagent. The sequence organization of the 749 bp internal control template obtained was summarized in Fig. 4.7.

The 749 nt ssRNA internal control template was transcribed *in vitro* using commercial transcription kit. The concentration of this purified ssRNA template was determined by the formula $A_{260}/0.025$ (µg/ml). A concentration of 140 ng/ul ssRNA template in one of the purified internal control stock solutions was detected by this method ($A_{260} = 0.035$), which equals $3.24 \times 10^{10}$ molecules/ul.

Detection limit of the internal control template was determined by RT-PCR reaction of 10 x serial ssRNA dilution with primer sets of SRV and AFCP. The results from the RT-PCR were illustrated in Fig. 4.8. The positive gel bands, a fragment of 576/575 bp in size, were seen at the $10^2$ to $10^7$ dilutions, but no band was observed at the
$10^8$ dilution reaction. Thus, the detection limit was 3,240 templates or 14 femtograms ssRNA internal control template.

**Multiplex RT-PCR in the presence of the internal control template.** A total of 64 specimens along with the ssRNA internal control template reagent as a co-amplification component were tested by multiplex RT-PCR reactions. The representative results are showed in Fig. 4.9, and a comparison of the results obtained from monoplex RT-PCR with the SRV primer set, direct EM, and multiplex RT-PCR as well as polyclonal AC-ELISA was summarized in Table 4.5.

Using ssRNA internal control as a template, the RT-PCR products with primer sets SRV and AFCP were 576 bp and 475 bp in length, respectively, while the positive RT-PCR products detected in the fecal samples by co-amplification with the same primer sets were 473 bp or 464 bp in size, respectively, which was easily differentiated based on their sizes.

The profile of multiplex RT-PCR results could be classified into three patterns (Fig. 4.9). Pattern 1, both IC and sample were positive, or sample was positive but IC was negative, was defined as positive; pattern 2, IC was positive but sample was negative, was defined as negative; pattern 3, neither IC nor sample were positive, was defined as inhibited. Based on that definition, of 64 controlled reactions tested, 8 were determined to be inhibited.
4.5 DISCUSSION

Until recently, the primary method to detect small round viruses including astrovirus in feces/gut contents was direct EM with negative staining. This conventional method is relatively sensitive enough to identify the agents since the patients or animals with diarrhea caused by astroviruses frequently shed large numbers of viral particles (26). The sensitivity of direct EM has been estimated to be $10^6$ to $10^7$ virus particles per gram of specimen (47). However, only 10% of viral particles may exhibit the five- or six-pointed star-like characteristic, so it is difficult sometimes to differentiate astroviruses from other featureless small round viruses. Our current results using direct EM tests of 319 field fecal samples showed that the positive rate of small round viruses was 32.2% (Table 4.4), similarly, the EM results from 64 samples, which were part of the 319 samples, and used as paired samples tested by other assays, released a 34.4% positive rate (Table 4.5), which included turkey astrovirus and other small round enteric viruses. Due to this limitation of EM, Reynolds (35) and others suggested the use of IEM techniques which is reliable and can enhance not only the specificity of the test but also the sensitivity if the corresponding antiserum is available (2, 20, 25, 35). However, IEM may mask the characteristic morphologic star feature or fail to detect new serotypes because of lacking specific antiserum, or existing cross reactions between different viruses. Generally, both direct EM and IEM techniques are time-consuming and labor-intensive, especially when handling large number of field specimens, and furthermore, the application of theses techniques needs sophisticated equipment and experienced personnel.
The AC-ELISA is a relatively quick and simple assay to detect the antigen of viral agents. An antigen capture EIA for detecting human astroviruses was developed by Herrmann et al. using a group-specific MAb (8E7) (14, 15). A modification of this assay using biotinylated detector antibody was developed by Moe C.L., et al. (34). Both EIAs were shown to be useful tools, especially when a large number of samples need to be tested during epidemiologic investigation (5, 16, 28, 34, 44). However the sensitivity of EIA is not very high, similar to IEM (10^5 to 10^6 viral particles per gram of stool) (15, 34). For turkey astrovirus detection, Koci et al. (21) analyzed the reactivity between a recombinant TAstV-2 capsid protein and a MAb against TAstV (produced by James Guy (10), North Carolina State University) by western blot, enzyme-linked immunosorbent assay, and immunofluorescence in transfected cells (unpublished data). They found that all tests were positive. The monoclonal AC-ELISA were developed in this study. The results obtained using monoclonal AC-ELISA demonstrated that the assay possessed very high specificity. However, the assay was only detected 0.06 µg of viral protein when the sensitivity as indicated by testing 2 x serial dilutions of purified TAstV2001 containing a predetermined concentration of viral protein. A monoclonal AC-ELISA developed by Hassan et al., (12) did not detect an IBDV virus in samples that had a titer of less than 5 x 10^3 EID_{50}. Sharma et al. (43) obtained similar results where the monoclonal AC-ELISA did not detect viral antigens in thymuses and bursas of experimentally infected chicks. The monoclonal AC-ELISA established in the current study could easily detect TAstV2001 and TAstV1987 in all gut homogenate samples with a titer of 5 x 10^2 EID_{50}, which were from turkey embryos inoculated with these viruses. To some extent, it seems that our AC-ELISA is more sensitive. It is necessary to point out, the MAbs used
in our system were cell culture supernatants which had low antibody titers as compared to those from ascites fluid that contains high level of antibodies. Our assay did not detect the viruses in any field fecal samples, which indicated that virus concentration in these fecal samples was under the detection limit of the monoclonal AC-ELISA. The low sensitivity of the monoclonal AC-ELISA might be attributed to the mono-specificity of binding. Improvements in the sensitivity of the monoclonal AC-ELISA might be achieved by pooling several MAbs directed against different epitopes (12), increasing the binding capacity of the MAbs. The use of avidin-biotin system to immobilize the MAbs which would result in greater capture capacity, might be another approach to improve the sensitivity of monoclonal AC-ELISA. Monoclonal AC-ELISA provides an alternative, practical method for the detection of astrovirus antigens after the viruses are enriched via culture in embryos.

Polyclonal AC-ELISA showed a 45.2%, 34.3%, and 20.2% higher positive rate than EM, monoplex RT-PCR, and multiplex RT-PCR, respectively. As shown in Table 4.5, the agreement between EM and polyclonal AC-ELISA was 85.3%, indicating the detection spectrum of EM is wider than the polyclonal AC-ELISA and the polyclonal AC-ELISA is more sensitive than EM. The higher positive detection rate of the polyclonal AC-ELISA over RT-PCR is due to its wider detecting spectrum than RT-PCR but not a higher sensitivity since 5% of the fecal samples detected positive by RT-PCR were not positive by the polyclonal AC-ELISA (data not shown). The polyclonal AC-ELISA can be used for diagnostic or epidemiologic purposes.

In addition to the above phenotyping methods, genotyping assays, such as molecular probes and reverse-transcriptase polymerase chain reaction (RT-PCR), were
recently developed to detect both human astrovirus and turkey astrovirus as the partial or complete gene or genomic sequence data became available (19, 22, 34, 50, 51). The sensitivity of probes is similar to enzyme immunoassay (EIA) (34, 50). But the sensitivity for RT-PCR is much higher compared to EIA. It was estimated to be 10 to 100 viral particles per gram of sample (42). For example, RT-PCR detected astroviruses in 32% of the samples tested, whereas EIA only had a detection rate of 10% in an investigation of an outbreak of astrovirus gastroenteritis at a day care center (33). At present, if more sequence data becomes available, it would be possible to design primer sets which can not only be used to identify multi-serotypes but also can differentiate between specific strains. For this purpose, Koci et al. (22) tried to design two sets of primers, one based a conserved polymerase region, and one from the variable region. For human astrovirus detection, a pan-reactive RT-PCR was developed, which had a good correlation with EIA. Unfortunately, this kind of test could not be developed for animal astrovirus identification since the similarity within/between the species of animal astroviruses is not high, and the sequence data published so far are limited. Currently, two turkey astrovirus complete genomic sequence data are available in databases. Based on these two available sequences, three sets of primers were designed, and used to develop monoplex RT-PCR and multiplex RT-PCR assays for the detection of turkey astroviruses. The application of the monoplex RT-PCR with primer set SRV demonstrated that this assay was very sensitive and specific in spite of a narrow detection spectrum. For example, it can only amplify the TAstV2001 isolate, but not the TAstV1987 and TAstV1997 (SRV p14) isolates. On the contrary, the monoplex RT-PCR with primer set AFCP could only amplify TAstV1987 and TAstV1997 (SRV p14), but
Monoplex RT-PCR with degenerate primers, covering the polymerase regions of turkey astrovirus genomes, and multiplex RT-PCR with both SRV and AFCP primer sets overcame the shortages of the above monoplex RT-PCR, and showed good specificity and a wider detection spectrum. The PCR and RT-PCR can specifically amplify minute quantities of nucleic acid, and have been widely used with great success in clinical diagnostics. However, they are complicated enzyme catalytic reactions that could be adversely affected by many factors, including a variety of defined and undefined inhibitors in sample materials (8, 40, 45, 54), resulting in lowering the sensitivity of the reactions by causing false negative results. To monitor and decrease the false negative results, a number of PCR and RT-PCR internal control standards have been established (56). In this study, one ssRNA internal control template carrying two sets of primers which can amplify different TAstV isolates was developed, characterized, and applied to the multiplex RT-PCR system. The results of multiplex RT-PCR with the internal control as co-amplification component released a test inhibition rate of 12.5%, which is different from previous reports (40, 45). This difference may be due to different specimens and RNA extraction methods used in our study. It is believed that the majority of inhibitors are small molecules. Therefore, the inhibitors can be removed through dialysis, or alternatively, through dilution, re-extraction of specimens as well as through virus culture to expand the virus titer. Afterwards, the retreated specimens can be retested. It was reported that a 1 to 6% additional positive rate was achieved using this procedure (40). Thus, the sensitivity of the RT-PCR can be increased. However, the internal control itself can cause false negative test results since it competes for the same reaction substrates as the target templates in the RT-PCR reaction. To eliminate this
adverse effect of the IC, the IC template should be added into the reaction system at a minimal level but enough for reliable amplification.

4.6 ACKNOWLEDGMENT

The authors thank R. N. Dearth for their technical assistance, and Ying Zhang (MCIC, OARDC) for sequencing. We wish to acknowledge Dr. H. John Barns at the college of Veterinary Medicine at North Carolina State University for providing initial fecal samples. We would like to thank Dr. Akbar Ali and Dr. Donald L. Reynolds, at the Department of Veterinary Microbiology and Preventive Medicine, Iowa State University for providing primer information. This work was supported by U.S. Egg and Poultry Association.
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30. **Marli, A. S.** personal communication.


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<table>
<thead>
<tr>
<th>Primer name</th>
<th>Length</th>
<th>Sequences, 5’ to 3’</th>
<th>Location</th>
<th>Polarity</th>
</tr>
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<td>AGGAATGACCAGACCAACTGGCAGAAGATTG</td>
<td>7103-7125</td>
<td>–</td>
</tr>
<tr>
<td>SRV-1-5*</td>
<td>26</td>
<td>TCTATTGGGATACAACAGGCAGGCGG</td>
<td>N/A</td>
<td>+</td>
</tr>
<tr>
<td>AFCP-F1</td>
<td>25</td>
<td>CACAAGGTGACAAACAGGATACAC</td>
<td>6094-6114</td>
<td>+</td>
</tr>
<tr>
<td>AFCP-R1</td>
<td>25</td>
<td>ATCCTTGATATGCTGTTTGAGAGTC</td>
<td>6529-6553</td>
<td>–</td>
</tr>
<tr>
<td>TAPG-L1</td>
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<td>TGGTGGTGYTTYCTCAARA</td>
<td>3835-3853, 3929-3945</td>
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</tr>
<tr>
<td>TAPG-R1</td>
<td>19</td>
<td>GYCKGTCATCMCCRTARCA</td>
<td>4414-4432, 4511-4529</td>
<td>–</td>
</tr>
</tbody>
</table>

**Table 4.1.** Primers used for RT-PCR. A indicates the position within the sequence of AF206663. B indicates the position within the sequence of Y15936. * SRV-1-3 and SRV-1-5 was designed by Dr. Akbar Ali and all others were designed at our lab.
Figure 4.1 Schematic diagram of the development of an internal control ssRNA template.
<table>
<thead>
<tr>
<th>Animal used for production of antisera</th>
<th>(\alpha)-T AstV1987</th>
<th>(\alpha)-T AstV2001</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Homo</td>
<td>Hetero(^A)</td>
</tr>
<tr>
<td>guinea pig</td>
<td>1</td>
<td>409,600</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>NA</td>
</tr>
<tr>
<td>chicken</td>
<td>1</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>NA</td>
</tr>
<tr>
<td>turkey</td>
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<tr>
<td></td>
<td>3</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>NA</td>
</tr>
</tbody>
</table>

**Table 4.2.** The AB-ELISA titers of polyclonal hyperimmune antisera produced in guinea pig, chicken or turkey against T AstV1987 or T AstV2001 purified with CsCl.

A. AB-ELISA was performed in the micro-titer plate pre-coated with CsCl purified T AstV2001 and hyperimmune antiserum against T AstV1987.

B. AB-ELISA was performed in the micro-titer plate pre-coated with CsCl purified T AstV1987 and hyperimmune antiserum against T AstV2001.

Homo is an abbreviation of homologous.
Hetero is an abbreviation of heterologous.
Table 4.3. Characteristics of monoclonal antibodies produced against TAstV2001.

A: Reaction/Cross reaction was determined by an AC-ELISA.
B. Ig isotype was determined by an AB-ELISA using a commercial MOUSE MONOCLONAL ANTIBODY ISOTYPING kit (Sigma) followed the instructions of manufacture.

a: TGDV = turkey group D rotavirus;
b: TGAV = turkey group A rotavirus;
c: TCV = turkey coronavirus (Minisota strain);
d: TEV = turkey enterovirus;
e: Wa-Rota = human group A rotavirus;
f: 202JP1 = turkey atypical rotavirus;

All viruses were from fecal samples except the Wa-Rota, which was propagated in cells.

NT = ‘not tested’.
<table>
<thead>
<tr>
<th>Hybridoma Cell secreting MAb</th>
<th>Reaction/Cross reaction with&lt;sup&gt;A&lt;/sup&gt;</th>
<th>Ig Isotype&lt;sup&gt;B&lt;/sup&gt;/Subisotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TAv1987</td>
<td>TAv1997</td>
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<td>1-11D</td>
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<td>+</td>
</tr>
<tr>
<td>2-10G</td>
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<td>+</td>
</tr>
<tr>
<td>4-9C</td>
<td>+</td>
<td>+</td>
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<td>1-5G</td>
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<tr>
<td>2-6D</td>
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<td>4-10E</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2-7A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2-10H</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3-8D</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2-8A</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 4.3.** Characteristics of monoclonal antibodies produced by TAv2001.
Figure 4.2. The partial viral particles detected by direct electron microscope in fecal preparation from the birds infected with PEMS. A-1 and A-2 are adenoviruses; B-1 to B-4 are turkey coronaviruses; C-1 and C-2 are small round viruses, which may include turkey astrovirus, enterovirus/calicivirus; D-1 and D-2 are rotaviruses; E is alfalfa-mosaic-like virus.
<table>
<thead>
<tr>
<th>Virus</th>
<th>EM: Positive/total samples</th>
<th>EM: positive %</th>
<th>RT-PCR(^a): positive %</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRV</td>
<td>103/319</td>
<td>32.2%</td>
<td>43.2%</td>
</tr>
<tr>
<td>TCV-like</td>
<td>98/319</td>
<td>30.7%</td>
<td>NT(^b)</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>304/319</td>
<td>95.6%</td>
<td>NT</td>
</tr>
<tr>
<td>Bacterial phage</td>
<td>309/319</td>
<td>96.8%</td>
<td>NT</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>49/319</td>
<td>15.4%</td>
<td>NT</td>
</tr>
<tr>
<td>Alfalfa mosaic-like</td>
<td>3/319</td>
<td>0.9%</td>
<td>NT</td>
</tr>
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</table>

**Table 4.4.** Frequency of viruses detected by direct EM and RT-PCR in fecal samples of poult affected by poult enteritis and mortality syndrome (PEMS).

\(^a\): Results were obtained by RT-PCR with primer set of SRV1-3 and SRV-1-5 for TAstV.

\(^b\): NT = ‘not tested’.
Figure 4.3. Representative results of the monoplex RT-PCR of 3 fecal samples with primers of SRV 1-3 and SRV-1-5. M = 100 bp DNA ladder; Lane a = TAstV2001; Lane b = TAstV1987; and Lane c = TAstV1997 (SRV p14).
Figure 4.4. Representative results of the monoplex RT-PCR of 3 fecal samples with primers of AFCP-F1 and AFCP-R1. M = 100 bp DNA ladder; Lane a = TAstV2001; Lane b = TAstV1987; and Lane c = TAstV1997 (SRV p14).
Figure 4.5. Representative results of the multiplex RT-PCR of 3 fecal samples with primers of SRV 1-3 and SRV-1-5 and AFCP-F1 and AFCP-R1. M = 100 bp DNA ladder; Lane a = TAstV2001; Lane b = TAstV1987; and Lane c = TAstV1997 (SRV p14).
Figure 4.6. Representative results of the RT-PCR of 3 fecal samples with degenerate primers TAPG-L1 and TAPG-R1 showed an expected 601 bp band. M = 100 bp ladder. Lane a: sample TAstV2001; lane b: TAstV1987; and lane c: TAstV1997 (SRV p14) is shown one product of 601 bp.
AGCTTGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCTGGAATTCGCTGTGCTTCTATTGGGATACAACACAGGCAAGCCGACCAAGGATACAGATACACAGGAAGCGGAACACGTAGAAAGCCACACGCCAAAGAGAAAGCAGGTAGCTTGCAGTGGGCTTACATGGCGATAGCTAGACTGGGCGGTTTTATGGACAGCGAAACCGGAATTGCCAGCTGGGGCGCCCTTGGTAAGGTTGGGAAGCCCTGCAAAGTAAACTGGATGGCTTTCTTGCCGCCAAGGATCTGATGGCGCAGGGGATCAAGATCTGATCAAGAGACAGGATGAGGATCGTTTCGATGTTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCAGCGGGTCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCA

Figure 4.7. The sequence organization of internal control ssRNA Template (749 bp in length).

**Black Bold** letters are HindIII restriction enzyme site; **Red bold** letters are T7 promoter sequence; **Blue bold** letters are linkers of pCR 2.1 vector; the insert fragment (brown) is located between the linkers, which is 601 nt in length, of which, 500 nt is from pCR 2.1, and 71 nt (Int-L1) is up-stream primer, and 70 nt is down stream primer (Int-R1); **Green bold** letters indicate restriction enzyme EcoR I site.

The internal control consists of 25% of A, 20.6% of T, 30% of G, and 24.4% of C (A=188 + T(U)=154 + G=224 + C=183 = 749 bp) with molecular weight (MW) of 2.6 x 10^5 Kd (188 x 347.2 + 154 x 324.2 + 224 x 363.2 + 183 x 323.2 = 2.6 x 10^5).
Figure 4.8. The results of determination of detecting limit of the internal control template of 10 x serial dilutions by RT-PCR with primer sets of SRV and AFCP. M = 100 bp DNA ladder; Lane a to f was observed a 576/575 bp positive bands in the $10^{-2}$ through $10^{-7}$ dilution reactions. Lane g is showing a negative result in the $10^{-8}$ dilution reaction.
Figure 4.9. Representative results of multiplex RT-PCR with internal control template for the detection TAstVs in fecal specimens. M = 100 bp DNA ladder; Lane a, c, and h are showing a 575/576 bp amplicon, indicating negative results; Lane b, d, and g are displaying 2 bands with the size of 576/575 bp and 464/473 bp, indicating both samples and IC are positive; Lane f is showing only sample is positive but not the IC; Lane e is showing double negative, indicating there existed inhibitor in the RT-PCR reaction system.
<table>
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<tr>
<th>Detecting method</th>
<th>Positive/ tested</th>
<th>Positive percent</th>
<th>With Monoplex RT-PCR match rate</th>
<th>With Multiplex RT-PCR match rate</th>
<th>With Polyclonal AC-ELISA Match rate</th>
<th>Higher detection percent than EM</th>
<th>Higher detection percent than Monoplex RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>EM</td>
<td>22/64&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34.4</td>
<td>54.5% (12/22)</td>
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<td>85.3%</td>
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<tr>
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<td>12/64</td>
<td>18.8</td>
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<td>16/64</td>
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</tbody>
</table>

**Table 4.5.** Comparison of the results detected by EM, monoplex RT-PCR, multiplex RT-PCR with internal control.

a: 64 fecal or gut contents samples were selected randomly from a total of 319 samples.
b: Monoplex RT-PCR was performed using primers of SRV-1-3 and SRV-1-5.
c: Multiplex RT-PCR was performed using primers of SRV-1-3 and SRV-1-5 and AFCP-F1 and AFCP-R1.
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infectious bursal disease, and Newcastle disease viruses in a single serum


