Enteric Viruses in Turkey Flocks: A Historic Review

ABSTRACT

In this review, diagnostic techniques and viral agents involved in enteric diseases affecting turkeys are described. Data from field observations and laboratory researches have been reported in turkey flocks for over 70 years, and several viruses have been identified. After a period of 30 years of inoculation experiments and neutralization studies, adequate visualization of the viruses was achieved using electronic microscopy. During the following years, several studies were then conducted to isolate and classify those viruses using cell-culture, embryo-propagation, serological tests, genome electropherotyping by polyacrylamide gel electrophoresis of double-stranded RNA viruses, and recently, nucleic acid studies. Thus, since the 1990s, the nucleic-acid technology has focused on genomic surveys and on the detection of specific segments of the genome of each virus using the polymerase-chain reaction, resulting in several prevalence studies and phylogenetic analyses of different isolates and proper classification of the viruses.

INTRODUCTION

Enteric disorders affecting turkey flocks were first reported in 1937 by Pomeroy and Fenstermacher, but only in 1950s it was frequently identified in poults. Reports were primarily based on clinical observations of the flocks and reproduction of the disease by inoculation of intestinal contents from affected flocks, neutralization tests, and physicochemical analysis of the intestinal filtrates. Although no etiological agent was identified until the end of the 1960s, those experiments helped to identify some physicochemical and cross-neutralization characteristics of the causative agent and the development of the disease on the birds.

Fujisaki et al. (1969) were the first to demonstrate the presence of reoviruses in the feces of poults with enteric disease using electronic microscopy (EM). During the next 10 years, adenoviruses, astroviruses, coronaviruses, picornaviruses and rotaviruses were also observed in the intestinal contents of turkeys with enteric disorders using EM.

Virus propagation in embryonated eggs and cell cultures was intensively evaluated between the 1960s and the 1990 in an attempt to standardize techniques for the propagation and isolation of each enteric virus. At the same time, specific serological techniques were developed, providing more detailed information on the antibody reactions to each virus.

The development of polymerase chain reaction (PCR) allowed rapid and specific detection of enteric viruses, and has been widely applied since the last decade for the diagnosis and survey of the prevalence of those viruses, as reported by several authors all over the world (McNulty et al., 1979a; Andral & Toquin, 1984a; Saif et al., 1985).
Around the same time, sequence analysis has described the DNA profile of circulating viruses, resulting in phylogenetic classification that helps understanding the distribution of the different virus strains, which is still a challenge for future studies.

**Early investigations on enteric diseases**

In 1937, Pomeroy and Fenstermacher identified an enteric disease affecting turkey flocks in the United States characterized by bloody diarrhea and increased mortality, which was called hemorrhagic enteritis (HE). In 1951, another intestinal disorder causing catarrhal enteritis, weight loss, and low mortality in turkeys was identified and was considered similar to the blue comb disease of chickens (Peterson & Hymas, 1951).

After these first descriptions, most studies on intestinal diseases until the end of the 1950s were used intestinal filtrate inoculation to reproduce the disease (Pomeroy & Sieburth, 1953; Sieburth & Johnson, 1957; Gross & Moore, 1967). Although this did not provide any information on the etiological agents, Sieburth & Johnson (1957) reproduced the so-called blue comb disease inoculating an antibiotic-treated filtrate of intestinal contents of affected flocks in young poults, and were the first to suggest that a virus could be the causal agent of this disease.

In 1968, the inoculation with *Vibrio* spp. cultures also reproduced the blue comb disease, and was called transmissible enteritis (Truscott, 1968). In 1969, however, Hofstad et al. did not demonstrate any pathogenicity associated with isolated vibrio. It was later shown that the blue comb disease agent passed through 0.22nm filters (Deshmukh et al., 1969; Hofstad et al., 1969) and could not be isolated in bacteriologic mediums, which led Adams & Hofstad (1971) to strongly suggest that the agent was a virus. Adams & Hofstad, in 1972, showed that different antibiotics were not able to significantly reduce the lesions in embryos inoculated with the disease, and that no signs of bacteria were observed under light microscopy.

These early investigations on enteritis that affected turkey flocks did not effectively demonstrate the causative agents, but several studies were carried out to try to describe the physical-ochemical properties of the isolates (Deshmukh et al., 1969; Fujisaki et al., 1969; Scott & McFerran, 1972; Domermuth & Gross, 1975).

Inoculation of embryos and cell cultures, together with serological methods and electron microscopy, were important tools for the isolation and identification of the etiological agents involved in those enteric disorders.

**Electron microscopy**

In 1969, reoviruses, papovavirus, and enterovirus were observed in turkeys affected with blue comb disease using electron microscopy (EM; Deshmukh et al., 1969), and in 1972 virus-like particles were also demonstrated in young turkeys and embryos affected with the same disease by Adams et al. (1972).

Since then, other enteric viruses were identified using EM. Coronavirus-like particles were observed in intestines of turkeys (Panigraphy et al., 1973) and embryos (Ritchie et al., 1973) inoculated with intestinal preparations from flocks affected with blue comb. Later, intranuclear viral particles were observed in the spleen and intestines of turkeys with HE (Carlson et al., 1974; Tolin & Domermuth, 1975), and these studies were the first to demonstrate that an adenovirus was the causal agent.

Rotavirus was first observed under EM in the feces of two- to three-week-old turkey poults suffering from enteritis and diarrhea (Bergeland et al., 1977) and stunted turkeys with diarrhea (McNulty et al., 1978). McNulty et al. (1979a) then validated the use of EM for the detection of enteric viruses (rotavirus, adenovirus, and enterovirus-like particles) directly from turkey and chicken feces. In 1980, astroviruses were detected for the first time in young turkeys presenting diarrhea and mortality (McNulty et al., 1980a).

In 1979, Imada et al. identified a picornavirus in the rectal contents of broilers chicks using EM and serological methods (neutralization and fluorescence), and found that its main target were the kidneys, and therefore, it was named "avian nephritis virus" (ANV). Andral et al. (1984b) later detected a picorna-like virus by EM in turkeys with enteric and respiratory signs.

Trampel et al. (1983) first reported parvovirus-like particles in the intestines of turkeys associated with enteropathy, showing intranuclear inclusions bodies in the epithelial cells of the ileum using EM, but, because the authors could not detect any vascular or cell damage in the intestines, it was classified as an enteropathy and not as enteritis.

The prevalence of enteric viruses then started to be studied using EM in several countries (McNulty et al., 1979a; Andral & Toquin, 1984a; Saif et al., 1985). Reynolds et al. (1987) demonstrated an association of different viruses (astrovirus, rotavirus, rotavirus-like viruses, atypical rotaviruses, enteroviruses, and reoviruses) in affected turkeys using EM, immune
electron microscopy, and electropherotyping of double-stranded RNA viruses.

**Cell culture and embryo inoculation**

Sieburth & Pomeroy (1955) were the first to demonstrate the propagation of the blue comb disease agent in yolk sac of embryonated chicken eggs, but only in 1971 (Adams & Hofstad, 1971) the disease was reproduced in turkeys using the agent propagated in 15-day-old embryos. Reoviruses were also isolated from turkeys affected with blue comb disease based on physical-chemical characteristics, its effects on chicken and turkey kidney cell culture, embryo inoculation, and neutralization tests (Fujisaka et al., 1969). Then, in 1969, Wooley et al. (1969) and Fujisaka et al. (1969) reproduced the disease in young turkeys inoculated with cell-propagated reoviruses, but not by Deshmukh et al. (1969), who used the same technique.

Wooley et al. (1972) distinguished two cell-propagated reoviruses, one pathogenic and one nonpathogenic, using sucrose density gradient centrifugation, that did not cross-reacted, and the nonpathogenic was considered ubiquitous in intestinal contents of turkeys.

Ahmed (1971) claimed to have isolated turkey adenovirus in cell culture followed by propagation in fertile turkey and chicken eggs; however, the author did not use serum neutralization test, and therefore the detected virus may have been a fowl adenovirus (McFerran et al., 1975). Scott and McFerran (1972) isolated turkey adenovirus serotype 1 (TAV-1) from respiratory, gastrointestinal, and urinary system of turkeys with conjunctivitis, nephritis and airsacculitis, using turkey kidney cell culture, and also isolated the agent from non-affected birds. The same agent was isolated from apparently healthy turkeys in United States, but when inoculated in poult, it caused significant growth depression and respiratory signs (Cho, 1976).

In 1988, fowl adenoviruses were isolated in chicken embryos, demonstrating that yolk sac can be an alternative to cell culture for any group I avian adenovirus, including turkey adenovirus (Cowen, 1988).

Fasina & Fabricant (1982) were the first to report HEV infection in spleen cell suspension cultures, but were not able to propagate the virus. In that same year, Nazerian & Fadly (1982) propagated HEV in lymphoblastoid B cells cultures derived from Marek’s disease tumor, and in 1990, turkey leukocyte cell culture was used to propagate the virus and produce an attenuated HE vaccine that protected turkeys against virulent HEV (van den Hurk, 1990).

Coronavirus was not successfully cultivated in cell culture (Deshmukh et al., 1973) and or detected in reovirus cultures (Fujisaki et al., 1969; Wooley et al., 1973). McNulty et al. (1980a) also had difficulties in propagating astroviruses in cell culture. Coronavirus was propagated on cell culture only in 1989 using continuous human rectal adenocarcinoma cell line enhanced with trypsin, showing that its cytopathic effect and pathogenicity for turkey poult was maintained after 5 passages (Dea et al., 1989).

McNulty et al. (1979b) were the first to propagate avian rotaviruses in chick kidney and chick embryo liver cell cultures from chickens and turkeys with signs of diarrhea and Castro et al. (1992) propagated rotavirus in seven day-old chicken embryos using liver, intestines and pancreas of turkeys from flocks exhibiting increased mortality, bloody droppings, and yolk retention.

Astrovirus was propagated in turkey embryos by Koci et al. (2000a), but no successful cell culture procedure was achieved.

**Serological investigations**

Since the first experiments with enteric diseases were conducted with inoculation of intestinal suspensions, serum neutralization tests were used. Different authors demonstrated the neutralizing effect of poult serum recovered from blue comb disease in young poult inoculated with an intestinal suspension of affected flocks (Sieburth & Johnson, 1957; Tumin & Pomeroy, 1958) and in HE outbreaks, preventing and reducing losses (Domermuth & Goss, 1975). The test was also used to demonstrate different serotypes of turkey adenovirus (Scott & McFerran, 1972; McFerran et al., 1975), rotavirus (McNulty et al., 1980b) and reovirus (Kawamura et al., 1965; Sahu & Olson, 1975; Wood et al., 1980).

As neutralization tests were considered time-consuming and expensive, because they depended on the development of infection in poult (Pomeroy et al., 1975), several other serological techniques were evaluated.

Direct immunoelectron microscopy was used to detect an antibody reaction specific to turkey coronavirus (TCoV) that did not cross-reacted with other coronaviruses species (Ritchie et al., 1973) and astroviruses in poult affected with enteritis and diarrhea and enteroviruses (Saif et al., 1990).
The first reports of immune techniques for HE diagnostic was the agar gel diffusion precipitin test (Domermuthet al., 1972) and the microimmunodiffusion test (Domermuth et al., 1973) that detected antibodies in the spleen of inoculated turkeys. Nevertheless, in 1977, an avirulent live vaccine against the hemorrhagic enteritis virus (HEV) was developed using the marble spleen disease virus of pheasants (Domermuth et al., 1977), which was considered harmless for turkeys, but showed cross-protection with HEV (Domermuth et al., 1977).

Patel et al. (1975) were the first to describe a fluorescent antibody test (FA) for coronavirus detection in the intestines of turkeys during the acute phase of the disease, but the test was not efficient for the detection of chronic carriers (Pomeroy et al., 1975). However, an indirect fluorescence antibody test later demonstrated antibodies in the serum of recovered birds (Patel et al., 1976). Both methods were considered useful for the recognition of field epirnithics of coronaviral enteritis (Patel et al., 1977).

A hemagglutination-inhibition test showed that there was no antigenic relationship between TCoV and other animal coronaviruses involved with enteric problems (Pomeroy et al., 1975) and it was considered an easy and applicable serological test by Dea et al. (1986). The tests of Pomeroy et al. (1975) were not able to produce an inactivated vaccine against coronaviruses infections.

Immunofluorescence studies were used to show intestinal lesions caused by coronaviruses in inoculated poults (Pomeroy et al., 1978) and to restrict the site of immunoglobulin secretion in intestines of recovered poults (Nagaraja & Pomeroy, 1980). This method was also used to demonstrate antibodies against avian nephritis virus in turkey flocks (Connor et al., 1987; Nicholas et al., 1988) and showed that avian rotavirus had a common antigen group with their mammalian counterparts that cross-reacted in many serological tests, thereby allowing the use of mammalian antisera for avian rotavirus detection (McNulty et al., 1979b).

An enzyme-linked immunosorbent assay (ELISA) was developed for the detection of antibodies against fowl adenovirus in 1980 (Dawson et al., 1980), TCoV in 1989 (Dea & Tijssen, 1989), and enterovirus in 1993 (Hayhow & Saif, 1993). And an association of this technique with an immunohistochemistry test demonstrated HEV distribution on organism and the pathogenesis of the disease in inoculated turkeys (Silim & Thorsen, 1981).

### Genome electropherotyping by polyacrylamide gel electrophoresis

Rotaviruses and reoviruses are double-stranded RNA viruses (dsRNA) and studies on the their pattern of migration on polyacrylamide gel electrophoresis (PAGE) were conducted to identify differences among isolates. Gouvea & Schnitzer (1982) studied several avian reoviruses isolates and demonstrated that they contain 10 dsRNA segments and that these showed different migration patterns on PAGE; however, these patterns were not related to differences in serotype.

This technique was also used to study avian rotaviruses from chickens (McNulty et al., 1981) and turkeys (Saif et al., 1985) and, according to the migration pattern on silver-stained PAGE, avian rotaviruses were classified into different electropherogroups (Kang et al., 1986; Todd & McNulty, 1986) and electropherotypes.

In 1992, PAGE was validated as a rapid and sensitive method for the diagnostic of dsRNA viruses, such as reoviruses and rotaviruses, when compared with EM (Lozano et al., 1992).

### Nucleic acid technology

Pathogenesis studies of enteric viruses were conducted using in-situ hybridization technique to detect adenoviruses (Suresh & Sharma, 1996), astroviruses (Behling-Kelly et al., 2002) coronaviruses (Verbeek et al., 1991) and reoviruses (Liu & Giambonne, 1997) directly from tissues using PCR and reverse transcriptase polymerase chain reactions (RT-PCR).

PCR and RT-PCR started to be considered as diagnostic methods when specific techniques for each agent involved in turkey enteric syndromes were standardized (Liu et al., 1997; Breslin et al., 1999; Hess et al., 1999; Xie et al., 1999; Koci et al., 2000b; Da Silva et al., 2008; Zsak et al., 2008; Buenger et al., 2009; Moura-Alvarez et al., 2013; Nuñez & Piantino Ferreira, 2013; Moura-Alvarez et al., 2014).

The simultaneous occurrence of enteric viruses has been reported around the world by prevalence surveys of these agents using EM, immune electron microscopy, and electropherotyping of double-stranded RNA viruses (McNulty et al., 1979a; Andral & Toquin, 1984a; Saif et al., 1985; Reynolds et al., 1987). However, the screening of those viruses had increased in the last 10 years due to the development of PCR(Villarreal et al., 2006; Pantin-Jackwood et al., 2007; Jindal et al., 2008; Pantin-Jackwood et al., 2008; Jindal et al., 2010).

Sellers et al. (2004) was the first to develop a multiplex reverse transcription-polymerase chain reaction diagnostic test for the simultaneous detection of...
of enteric viruses in intestines and feces of affected turkeys and identified turkey astrovirus-2 (TAstV-2) and coronavirus (TCoV). Day et al. (2007) later developed a multiplex RT-PCR test for the detection and differentiation of turkey astrovirus-1 (TAstV-1), TAstV-2, ANV, Chicken astrovirus (CAstV), and Rotavirus in turkey and chicken samples.

RT-PCR was first evaluated by Spackman et al. (2005) for the detection of TAstV-2, TCoV, and avian reoviruses in the intestinal contents of turkeys affected with enteritis, and it is a more detailed test than the traditional PCR.

Sequences and phylogenetic analyses were conducted by several authors, demonstrating the genomic variability among isolates. Liu et al. (1997) showed that nucleotide sequence analysis was a sensitive and precise method for the differentiation of avian reovirus isolates. Then, differences between the nucleotide sequence of the S3 (Kapczynski et al., 2002) and S2 genes (Sellers et al., 2004) from turkeys, when compared with chicken and duck isolates were demonstrating, suggesting their classification in a different subgroup, the Orthoreovirus genus.

Breslin et al. (1999) and Cavanagh et al. (2001) showed close genetic relationship between TCoV and infectious bronchitis virus, and Guy (2000) suggested a classification in the group 3 coronavirus, as confirmed by Gomaa et al. (2008) by the analysis of the complete genome of TCoV.

Imada et al. (2000) sequenced the avian nephritis virus, which was classified as a member of the family Astroviridae, and this the first avian astrovirus which genome was completely sequenced, showing close similarity to the turkey astrovirus. Koci (2000a) then isolated and sequenced the astrovirus obtained from the intestinal contents of young turkeys affected with poultry enteritis and mortality syndrome.

The sequence of turkey and chicken rotavirus VP8 was analyzed to verify if it was similar to a rotavirus isolated from calf feces (Rohwedder et al., 1995). HEV was completely sequenced by Pitcovski et al. (1998), but it was Davison et al. (2003) that later characterized avian and mammalian adenoviruses.

Once the genomic variability of a virus is identified, pathogenesis studies should be conducted to detect differences in the pathogenicity of the variant agent, as performed by Pantin-Jackwood et al. (2008). The authors conducted a pathogenesis study of 2 different TAstV-2 with variant capsid genes that produced an enteric disease in turkeys similar to non-variant known viruses. The viruses were inoculated and clinical signs, and gross and microscopic lesions were observed. Virus distribution in the organs was determined using immunohistochemistry, in-situ hybridization, and RT-PCR.

The International Committee on taxonomy of viruses (ICTV) stated that proper classification of viruses in the appropriate order and family should be conducted based on the molecular characteristics of the isolate (van Regenmortel et al., 2000).

CONCLUSION

Throughout the years, increasingly sensitive, rapid, and specific techniques were developed for the diagnosis of enteric viruses. The early studies using inoculation allowed a better understanding of enteric diseases. The emergence of, electron microscopy, cell culture, embryo-propagation, and serological tests, although expensive and time-consuming, provided essential information on the characteristics of the agents and of the disease.

The development of the PCR method brought the research and diagnostic of enteric viruses to a new level due to its specificity and speed, and therefore it is extensively used in prevalence surveys.

Studies on virus genome aid both to identify critical pathogenesis issues and to provide accurate classification of enteric disease viral agents according to their genomic variability.

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