Viral Agents Associated with Outbreaks of Diarrhea in Turkey Flocks in Quebec

S. Dea and P. Tijssen*

ABSTRACT

The relative importance of various enteric viruses associated with diarrhea of turkey poults was investigated by an evaluation of specimens received since 1982. Specimens originated from one to eight week old turkey poults, with mild to severe diarrhea, from 114 flocks in 42 commercial operations located in southern Quebec. The acute phase of enteritis occurred usually in poults between two and four weeks of age. Clarified intestinal contents were examined by direct electron microscopy and enzyme immunoassays. Enzyme-linked immunosorbent assays were performed with antisera to bovine rotavirus group antigen, avian reovirus types 1 to 5, and the prototype strain of the turkey enteric coronavirus. The presence of viruses could be demonstrated by electron microscopy in 55.3% of the specimens, and at least five different viruses were incriminated either alone or in combination. The coronavirus was by far the most common enteric virus with a prevalence of 47.5%. By enzyme-linked immunosorbent assay, rotavirus, reovirus and turkey coronavirus were detected in 14.5%, 18.1% and 61.4% of the specimens, respectively. By electron microscopy, 56.6% of these cases were positive for at least one virus.

RÉSUMÉ

Cette étude consistait à évaluer l'importance relative des divers virus associés à la diarrhée des dindonneaux; elle impliquait l'examen des échantillons soumis depuis 1982, lesquels provenaient de dindonneaux âgés d'une à huit semaines et issus de 114 élevages reliés à 42 exploitations commerciales du sud du Québec. La phase aiguë de la diarrhée affectait ordinairement des sujets âgés de deux ou quatre semaines. On examina du contenu intestinal clarifié, par la microscopie électronique, ainsi qu'au moyen d'épreuves immunoenzymatiques où on utilisait des antisérums spécifiques contre le rotavirus bovin, les sérotypes #1 à #5 du réovirus aviaire et la souche du prototype du coronavirus entérique de la dinde. La microscopie électronique permit de démontrer des particules d'au moins cinq familles virales, seules ou en association, dans 55,3% des échantillons. Le coronavirus s'avéra de loin l'entérovirus le plus fréquent, puisqu'il afficha une prépondérance de 47,5%.

Les épreuves immunoenzymatiques précitées permirent de détecter les antigènes de groupe des rotavirus et des réovirus, ainsi que les antigènes du coronavirus de la dinde, dans respectivement 14,5%, 18,1% et 61,4% des échantillons soumis au cours des deux dernières années. La microscopie électronique révéla la présence d'au moins un virus, dans 56,6% de ces échantillons.

INTRODUCTION

Diarrhea in turkey flocks is a complex problem resulting from one or several infectious agents, compounded by a lack of immunity and inappropriate management procedures. Although many viruses have been observed by direct electron microscopy of turkey and chicken fecal specimens, only coronaviruses and adenoviruses are established causes of various diarrheal syndromes in poultry (1,2). Other viruses, either alone or in combination, isolated from diarrheal specimens include astroviruses (3,4), caliciviruses (5), reoviruses (6-9), parvoviruses (10,11) and enteroviruses (4,9). More recently, the isolation and characterization of rotaviruses and pararotaviruses associated with a diarrhea syndrome characterized by increased mortality in two to three week old turkey poults have been reported (4,9,12,13).

Sudden or repeated outbreaks of mild to severe diarrhea in young turkey poults were experienced by a number of commercial turkey producers in Quebec during the past few years. This investigation was undertaken in order to find possible causative viral agents of these outbreaks. The reliability of the results obtained by direct electron microscopy (EM) and enzyme-linked immunosorbent assay (ELISA) was compared for the detection of enteric coronaviruses, rotaviruses and reoviruses in clinical specimens.

MATERIALS AND METHODS

CLINICAL SPECIMENS

Pooled intestinal contents from one to eight week old turkey poults from

*Centre de Recherche en Médecine Comparée, Institut Armand-Frappier, 531 boulevard des Prairies, Laval, Quebec H7V 1B7.

Reprint requests to Dr. P. Tijssen.

This study was partly supported by a grant received from the Medical Research Council of Canada and La Fédération des Producteurs de Volailles du Québec.

Submitted June 7, 1987.

114 commercial flocks in 42 operations located in southern Quebec, were submitted to our laboratory over a four year period for virological examination. These flocks were experiencing outbreaks of diarrhea, associated with significant variation in poult size, and an average mortality rate of 5.4%. The acute phase of enteritis was usually observed at an age of two to four weeks.

The specimens were homogenized in ten volumes of 0.05 M Tris-HCl buffer, (pH 8.0) and clarified by centrifugation at 5000 x *g* for 30 min at 4°C. The supernatants were then passed through Millipore membrane filters of 450 nm pore size and stored at -70°C until tested.

ELECTRON MICROSCOPY

Clarified intestinal contents were ultracentrifuged at 100,000 x *g* (Beckman L5-65 ultracentrifuge, SW50 rotor, Beckman, Palo Alto, California) for 2.5 h at 4°C through a 1 to 2 mL cushion of 30% sucrose (w/v). Pellets were then resuspended in 0.1 mL of distilled water and one drop of this suspension was placed on 200-mesh formvar carbon-coated grid. The material was stained with a 2% solution of phosphotungstic acid, pH 6.5, as described previously (14,15).

EGG INOCULATION

About 0.2 mL of clarified clinical specimens was inoculated into the amniotic cavity of 22 to 24 day old embryonated turkey eggs obtained from a source known to be free from all the usual specific pathogens of turkeys. After inoculation, the eggs were incubated at 37°C for three to four days. The intestines of the embryos were then fixed in 10% neutral buffered formalin and processed for paraffin tissue sectioning according to conventional methods. Sections were stained with hematoxylin-phloxin-safran (HPS), as described previously (16).

ANTISERA

Hyperimmune sera were obtained after immunization of guinea pigs and rabbits with turkey coronavirus ("Minnesota strain, Bluecomb agent"), which was serially propagated in embryonated turkey eggs and purified by ultracentrifugation on sucrose gradients as described elsewhere (17,18).

Rabbit and guinea pig antisera were also produced against the NCDV strain of bovine rotavirus and avian reoviruses types 1 to 5 (obtained from the American Type Culture Collection, Rockville, Maryland), which were cultivated, respectively, in African green monkey kidney (MA104) and primary chicken embryo kidney cells (7,19). Each virus was concentrated and purified following freon extraction and ultracentrifugation on CsCl gradients, according to Spence *et al* (20). Animal immunizations were done as described previously (21). In this study a pool of antisera to serotypes 1 to 5 was used in case of avian reoviruses.

ENZYME-LINKED IMMUNOSORBENT ASSAYS

Indirect ELISA tests were performed using modifications of previously described methods (22-24). Flat bottom polystyrene microtiter plates (Flow Laboratories, Inc., Mississauga, Ontario) were used as solid-phase adsorbents. The optimum dilutions of all reagents were determined by checkerboard titrations. A total reaction volume of 100 μL was used in all microtiter wells. All washes were performed five times with 0.05 M Tris-saline, pH 7.6 (TBS), containing 0.05% Tween 20 (TBS-T).

Plates were first coated with the guinea pig hyperimmune sera diluted 1:500 in 0.05 M sodium carbonate-bicarbonate buffer, pH 9.6. After incubation for 16 h at 4°C, the coated plates were treated with 1% bovine serum albumin (BSA grade V, Sigma) in TBS for 1 h, dried, and stored refrigerated until required. The solid-phase antibody was subsequently incubated for 3 h at 37°C with tenfold dilutions of the clarified fecal samples in TBS-T. Plates were then washed and incubated for 2 h at 37°C with the rabbit hyperimmune serum diluted 1:1000 with the same buffer. After a further washing step, the enzyme was introduced in the immune complex by incubation for 1 h at 37°C with peroxidase-conjugated goat antirabbit IgG antibodies (Boehringer Mannheim Biochemicals, Dorval, Quebec) diluted 1:1000 with TBS-T. After washing, the enzyme substrate solution, containing 1 mg/mL of 5-aminosalicylic acid and 0.005% hydrogen peroxide (pH 6.0) was added. After the reaction had proceeded for 45 min at room temperature, the optical density at 474 mm (OD474) was determined for each specimen using a spectrophotometer (Titertek Multiskan, Flow Laboratories, Inc., Mississauga, Ontario). A sample was scored positive if its average OD474 value was at least three times higher than that of the negative controls.

RESULTS

During the last four years, specimens from 83 out of 114 flocks tested were examined by both EM and ELISA, whereas samples from the remaining 31 flocks were examined by EM only.

The annual incidence of the various enteric viruses detected by EM in samples from diarrheic turkey poults is shown in Table I. Coronavirus-like particles, reoviruses, enteroviruses, adenoviruses and rotaviruses were observed either alone or in combination in 55.3% of all samples received between January 1983 and January 1987. Coronavirus-like particles were detected in 55 out of 114 flocks (47.5%), and represented by far the most common agent associated with the diarrheal outbreaks. In the last two years, coronaviruses were demonstrated in 44 out of 78 specimens (56.4%) submitted for virological examination. A prevalence of less than 5% was demonstrated for the others viruses involved.

Viral particles considered as coronavirus were moderately pleomorphic, but mostly spherical in shape (Fig. 1 A,B). They were enveloped and surrounded by a double fringe of regularly spaced petal-shaped projections, 12 to 17.4 nm in length, attached to the particles by a short thin stalk. The diameter ranged from 48 to 230 nm, with an average of 120 nm.

The morphology of the other viruses detected is illustrated in Fig. 2. Rotaviruses (Fig. 2A) varied in size from 57-65 nm depending on the presence of the outer capsid, whereas reoviruses (Fig. 2B) averaged 75-80 nm in diameter. These two viruses

TABLE I. Incidence of Enteric Viruses Detected in Diarrheic Poults from 114 Flocks by Electron Microscopy

Virus	No. of Positive Flocks in 1983	1984	1985	1986	Total (%)
Corona-like	1	7	18	23	49 (43.0)
Reo	1	3	0	0	4 (3.5)
Adeno	0	1	0	0	1 (0.8)
Rota	0	0	1	1	2 (1.7)
Entero	0	0	1	0	1 (0.8)
Corona + Reo	0	2	1	0	3 (2.1)
Corona + Entero	0	0	1	0	1 (0.8)
Corona + Rota	0	0	1	0	1 (0.8)
Corona + Entero + Adeno	0	1	0	0	1 (0.8)
Negative	5	15	17	14	51 (44.7)
Total submitted	7	29	40	38	114

[a]Submitted from January 1983 to January 1987

were distinguished on the basis of outer capsid layer morphology; in the case of rotaviruses, the arrangement of the capsomers of the inner capsid and the well defined outer layer provides the characteristic spoke-like appearance, whereas the outer layer of reovirus particles is featureless (25). Enteroviruses (Fig. 2C) were classified on the basis of size (average of 30 nm in diameter), their circular profile, and absence of any surface details. Many empty particles, with dark staining center were also noted. Adenoviruses (Fig. 2D) were of typical morphology and were present infrequently and then only in very small numbers.

Clarified intestinal contents containing numerous coronavirus-like particles were inoculated into the amniotic cavity of embryonated turkey eggs in order to study their pathological significance. Upon the first passage, there were very few deaths of the embryos, but gross lesions could be demonstrated in most of them. The duodenum, jejunum and ceca were markedly distended, with watery, greenish contents. The other organs appeared normal.

Histopathological lesions observed in the intestinal tract of embryos inoculated with four different coronavirus-like isolates were essentially similar. Lesions were most distinct in the jejunum but were also seen in the duodenum, ileum and ceca. Intestinal villi were shorter than normal and columnar absorptive cells were replaced by cuboidal or simple squamous epithelial cells. Fusion of the adjacent villi occurred occasionally. The lamina propria was infiltrated with mononuclear cells and denudation was sometimes observed.

Viral particles, morphologically indistinguishable from those detected from original clinical specimens, were detected by EM in clarified intestinal contents from infected embryos. The viral particles banded at a buoyant density of 1.18-1.20 g/mL in sucrose gradients (Fig. 1C). Immune aggregates were observed by IEM, when aliquots of rabbit hyperimmune serum produced against the Minnesota strain of turkey coronavirus were reacted with clarified intestinal contents of those embryos (Fig. 1D). No aggregates were observed with the intestinal contents of mock infected turkey embryos or with infected intestinal contents incubated with the rabbit preimmune serum.

In Table II, the results of ELISA and EM tests for the detection of coronavirus, rotavirus and reovirus, show that there was close agreement between the two tests in the case of specimens that were shown by EM to contain viral particles. However, ten

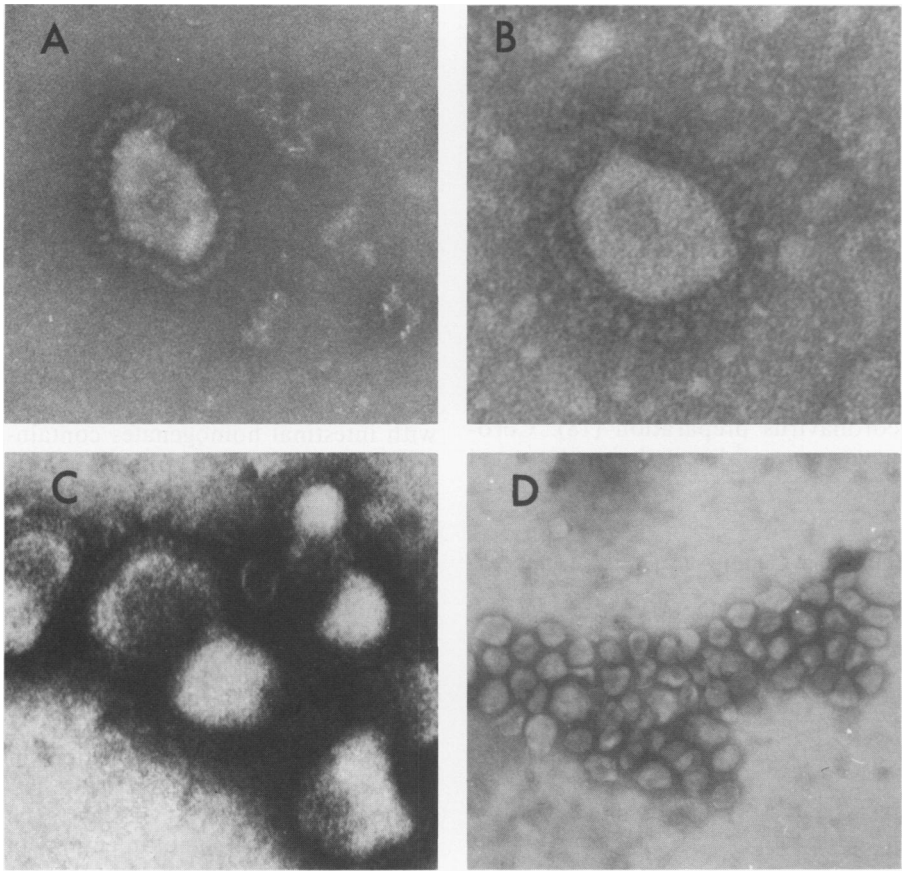

Fig. 1. Transmission electron micrograph of coronavirus-like particles recovered from intestinal contents of turkey poults with mild to severe diarrhea. A: A typical viral particle with regularly spaced petal-shaped projection. X185,000. B: A double fringe of peplomers was observed on a few particles. X210,000. C: Purified viral particles observed in fractions of sucrose gradients corresponding to buoyant density of 1.18-1.20 g/mL. X185,000. D: Aggregate demonstrated by IEM. X45,000.

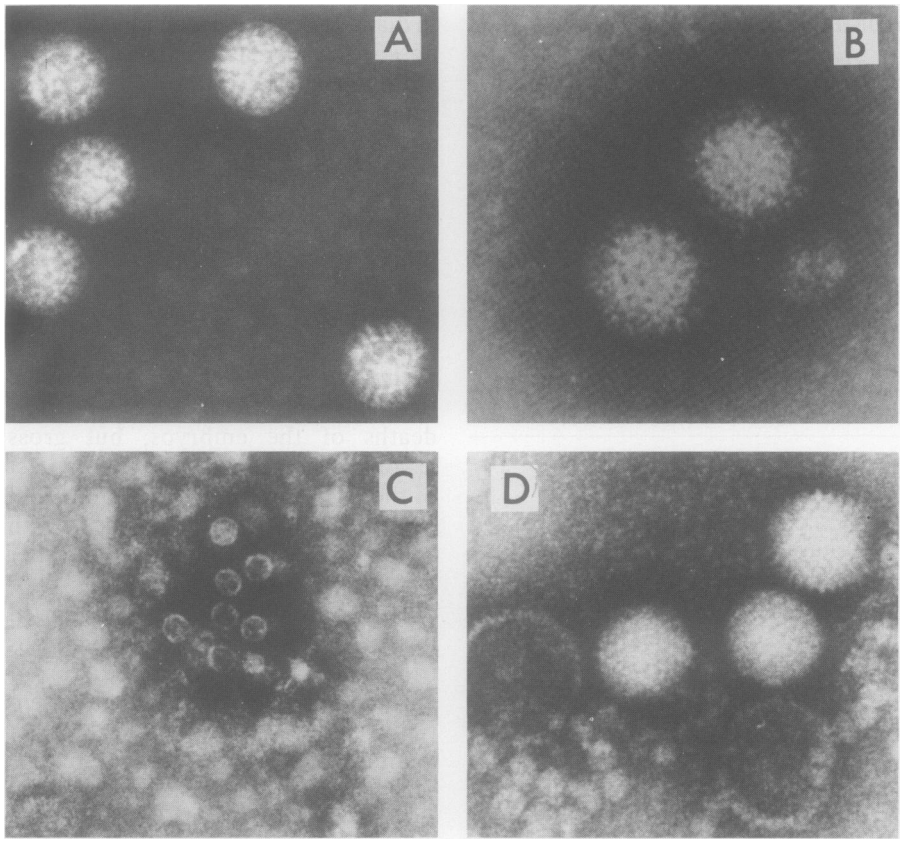

Fig. 2. Other viral particles that were associated with field cases of epidemic diarrhea in turkey poults. A: Rotaviruses. X185,000. B: Reoviruses. X220,000. C: Enteroviruses. X110,000. D: Adenoviruses. X150,000.

specimens that were negative by EM, were positive by ELISA for turkey coronavirus, while group antigens of reoviruses and rotaviruses were detected, respectively, in eight and five of these fecal samples. Overall, 61.4% (51/83) of the flocks tested by both techniques were positive for turkey coronaviruses, whereas 18.1% (15/83) and 14.5% (12/83) were positive for reoviruses and rotaviruses, respectively.

The detection limit of the ELISA with rabbit hyperimmune serum was investigated using serial dilutions of fecal extract and a purified turkey coronavirus preparation (18). Coronavirus was detected in the fecal extract diluted 1:2000, corresponding to approximately 10^3 coronavirus particles per mL as determined by EM. A 1:1000 dilution of the rabbit antiturkey coronavirus, antirotavirus and antireovirus antiserum was used throughout the test.

TABLE II. Viruses Detected by EM and ELISA in Intestinal Contents of Diarrheic Poults[a]

			ELISA					
			Rota		Reo		Corona	
Virus Detected by EM	No.	(%)	+	–	+	–	+	–
Corona only	38	(45.8)	5	33	3	35	36	2
Corona + Reo	2	(2.4)	0	2	2	0	2	0
Corona + Entero	1	(1.2)	0	1	0	1	1	0
Corona + Rota	1	(1.2)	1	0	0	1	1	0
Entero only	1	(1.2)	0	1	1	0	0	1
Rota only	2	(2.4)	1	1	1	2	0	2
Adeno only	1	(1.2)	0	1	0	1	0	1
Adeno + Corona + Entero	1	(1.2)	0	1	0	1	1	0
Negative	36	(43.4)	5	31	8	28	10	26
Total (%) positive			(14.5)		(18.1)		(61.4)	

[a]From June 1984 to January 1987

DISCUSSION

Diarrhea in turkey poults is becoming a serious problem for commercial turkey producers in Quebec due to increased mortality or serious weight loss in one to three week old poults. Within recent years, many flocks in southern Quebec have experienced outbreaks of diarrhea in young turkey poults, but there have been few investigations of the etiological agents associated with these enteric disorders.

In the present study EM and ELISA were used for virological investigations, and their efficiency was compared for routine examination of pathological specimens, because of their relative simplicity and speed. The results obtained revealed a high frequency of viruses associated with these outbreaks. By both techniques, it was shown that most often a coronavirus was involved. This agent was antigenically indistinguishable by IEM and ELISA to the transmissible enteritis virus (Bluecomb agent) of turkeys, originally isolated in Minnesota, and it had a similar buoyant density (26,27). Lesions induced in embryonated turkey eggs inoculated with intestinal homogenates containing numerous coronavirus-like particles were similar to those described previously (17,27,28).

Other viruses which were observed alone or in combination with the coronavirus included reoviruses, rotaviruses, enteroviruses and adenoviruses. The pathological significance of many of the viruses detected in avian intestinal contents remains to be determined. With the exception of coronaviruses and adenoviruses, their pathogenicity in turkeys needs to be verified by experimental infections in susceptible poults. A few recent reports have dealt with the etiological role of rotavirus strains isolated from outbreaks of diarrhea in chickens and turkeys (9,13,29). More recently, Saif

et al (4) were able to demonstrate the pathogenicity of turkey rotaviruses lacking the group antigen of conventional rotaviruses, which were shown to be involved in outbreaks of enteritis in the United States. Enteroviruses and reoviruses, although frequently detected in feces have not generally been associated with diarrhea in avian species (4,8,9,30,31).

The ELISA proved to be a reproducible, rapid and quantitative method for the detection of coronavirus, rotavirus and reovirus antigens in fecal samples of diarrheic poults. Particularly, the prevalence of rotavirus was higher by ELISA than by EM. The high sensitivity of the ELISA for detection of rotavirus group antigens in fecal samples has been frequently reported in the literature (22-24). The microtiter procedure is particularly suited for the large-scale testing of field samples.

ACKNOWLEDGMENTS

The technical assistance of M. Jacques Beaubien is gratefully acknowledged.

REFERENCES

1. **POMEROY BS.** Coronaviral enteritis of turkeys. In: Hofstad MS, Barnes HJ, Calnek BW, Reid WM, Yoder HW, eds. Diseases of Poultry. 8th ed. Ames, Iowa: Iowa State University Press, 1984: 553-559.
2. **DOMERMUTH CH, GROSS WB.** Hemorrhagic enteritis. In: Hofstad MS, Barnes HJ, Calnek BW, Reid WM, Yoder HW, eds. Diseases of Poultry. 8th ed. Ames, Iowa: Iowa State University Press, 1984: 511-516.
3. **McNULTY MS, CURRAN WL, McFERRAN JB.** Detection of astroviruses in turkey feces by direct electron microscopy. Vet Rec 1980; 106: 561.
4. **SAIF LJ, SAIF YM, THEIL KW.** Enteric viruses in diarrheic turkey poults. Avian Dis 1985; 29: 798-811.
5. **WYETH PJ, CHETTLE NJ, LABRAM J.** Avian calicivirus. Vet Rec 1981; 109: 477.
6. **DESHMUKH DR, POMEROY BS.** Avian reovirus. I. Isolation and serological characterization. Avian Dis 1969; 13: 239-243.
7. **NERSESSIAN BN, GOODWIN MA, KLEVEN SH, PESTI D.** Studies on orthoreoviruses isolated from young turkeys. I. Isolation and characterization. Avian Dis 1985; 29: 755-767.
8. **NERSESSIAN BN, GOODWIN MA, PAGE RK, KLEVEN SH.** Studies on orthoreoviruses isolated from young turkeys. II. Virus distribution in organs and serological response of poults inoculated orally. Avian Dis 1985; 29: 963-969.
9. **ANDRAL B, TOQUIN D, L'HARIDON R, JESTIN A, METZ MH, ROSE R.** Les diarrhées du dindonneau: un bilan des recherches virales effectuées (rotavirus, reovirus, adenovirus, pseudopicornavirus). Avian Pathol 1985; 14: 147-162.
10. **TRAMPEL DW, KINDEN DA, SOLORZANO RF, STOGSDILL PL.** Parvovirus-like enteropathy in Missouri turkeys. Avian Dis 1983; 27: 49-54.
11. **KISARY J, NAGY B, BITAY Z.** Presence of parvoviruses in the intestine of chickens showing stunting syndrome. Avian Pathol 1984; 13: 339-343.
12. **McNULTY MS, ALLAN GM, TODD D, McFERRAN JB.** Isolation and cell culture propagation of rotaviruses from turkeys and chicken. Arch Virol 1979; 61: 13-21.
13. **YASON CV, SCHAT KA.** Isolation and characterization of avian rotaviruses. Avian Dis 1985; 29: 500-508.
14. **DEA S, ROY RS, BEGIN ME.** Counterimmunoelectroosmophoresis for detection of neonatal calf diarrhea coronavirus: methodology and comparison with electron microscopy. J Clin Microbiol 1979; 10: 240-244.
15. **DEA S, ROY RS, ELAZHARY MASY.** Virus-like particles, 45 to 65 nm, in intestinal contents of neonatal calves. Can J Comp Med 1983; 47: 88-91.
16. **LAPIERRE J, MARSOLAIS G, PILON P, DESCOTEAUX JP.** Preliminary report on the observation of a coronavirus in the intestine of the laboratory rabbit. Can J Microbiol 1980; 26:1204-1208.
17. **DESHMUKH DR, LARSEN CT, POMEROY BS.** Survival of bluecomb agent in embryonating turkey eggs and cell cultures. Am J Vet Res 1973; 34: 673-675.
18. **DEA S, MARSOLAIS G, BEAUBIEN J, RUPPANNER R.** Coronaviruses associated with outbreaks of transmissible enteritis of turkeys in Quebec: hemagglutination properties and cell cultivation. Avian Dis 1986; 30: 319-326.
19. **ARCHAMBAULT D, ROY RS, DEA S, ELAZHARY MASY.** Comparative study of bovine rotavirus isolates by plaque assay. Can J Comp Med 1984; 48: 286-289.
20. **SPENCE L, FAUVEL M, PETRO R, BABIUK LA.** Comparison of rotavirus strains by hemagglutination-inhibition. Can J Microbiol 1978; 24: 353-356.
21. **BEGIN ME, DEA S, ROY RS, DAGENAIS L.** Comparison of electron microscopy, counterimmunoelectroosmophoresis and virus isolation in cell culture for the detection of bovine rotavirus. Proc 2nd Int Symp Neonatal Diarrhea. Saskatoon, 1978: 273-285.
22. **YOLKEN RH, KIM HW, CLEM T, WYATT RG, KALICA AR, CHANOCK RM, KAPIKIAN AZ.** Enzyme-linked immunosorbent assay (ELISA) for detection of human reovirus-like agent of infantile gastroenteritis. Lancet 1977; 2: 263-266.
23. **TIJSSEN P.** Practice and Theory of Enzyme Immunoassays. Amsterdam: Elsevier Biomedical Press, 1985.
24. **BELLAMY K, GARDNER PS, HAMBLING MH, RICE S, BRADBURNE AF.** Enzyme-linked immunosorbent assay for the detection of human rotavirus in stools. J Virol Methods 1983; 7: 65-72.
25. **PALMER EL, MARTIN ML, MURPHY FA.** Morphology and stability of infantile gastroenteritis virus: comparison with reovirus and bluetongue virus. J Gen Virol 1977; 35: 403-414.
26. **KING DJ.** Comments on the etiology and immunity of transmissible (coronaviral) enteritis of turkeys (Bluecomb). Am J Vet Res 1975; 36: 555-556.
27. **PANIGRAPHY B, NAQI SA, HALL CF.** Isolation and characterization of viruses associated with transmissible enteritis (Bluecomb) of turkeys. Avian Dis 1973; 17: 430-438.
28. **POMEROY KA, PATEL BL, LARSEN CT, POMEROY BS.** Combined immunofluorescence and transmission electron miscroscopic studies of sequential intestinal samples from turkey embryos and poults infected with turkey enteritis coronavirus. Am J Vet Res 1978; 39: 1348-1354.
29. **McNULTY MS, ALLAN GM, McCRACKEN RM.** Experimental infection of chickens with rotaviruses: clinical and virological findings. Avian Pathol 1983; 12: 45-54.
30. **KIBENGE FSB, GWAZE GE, JONES RC, CHAPMAN AF, SAVAGE CE.** Experimental reovirus infection of chickens: observations on early viraemia and virus distribution in bone marrow, liver and enteric tissues. Avian Pathol 1985; 14: 87-98.
31. **McNULTY MS, CURRAN WL, TODD D, McFERRAN JB.** Detection of viruses in avian feces by direct electron microscopy. Avian Pathol 1979; 8: 239-247.