

# TOROVIRUSES OF ANIMALS AND HUMANS: A REVIEW

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- I. Introduction
- II. Viruses
  - A. Biological Characteristics
  - B. Physical and Chemical Properties
  - C. Genome Organization and Replication
  - D. Protein Composition and Antigenic Determinants
- III. Clinical Features
  - A. Experimental Infection
  - B. Natural Infection
- IV. Pathology and Pathogenesis
- V. Immunity
  - A. Antigenic Relationships among Toroviruses
  - B. Immune Response in the Infected Host
- VI. Epidemiology
  - A. Seroepidemiology
  - B. Disease Association
  - C. Transmission
- VII. Diagnosis
  - A. Virus Detection
  - B. Antibody Detection
- VII. Taxonomy
- IX. Future Research
- References

## I. INTRODUCTION

Toroviruses are a group of enveloped positive-stranded RNA viruses that cause enteric, respiratory, and perhaps generalized infections in animals and humans. Their name refers to their unique morphological



features: An elongated bacilliform core with two rounded ends is surrounded by a membrane which may either tightly adhere to or "shrink-wrap" it without respecting the capsid's rod shape; in the first instance, straight or curved rhabdovirus-like particles are formed, whereas in the latter a biconcave disk results. There is little doubt that solitary torovirions have often been seen by electron microscopists, but their pleomorphism precluded their identification as viruses.

The equine and bovine toroviruses are the best-studied members of this new genus within the family Coronaviridae (Table I). Torovirus history is brief: The first representative, Berne virus (BEV), was isolated in Berne, Switzerland, in 1972 from a rectal swab taken from a horse with diarrhea 1 week before it died (Fig. 1A) (Weiss *et al.*, 1983). BEV is the only equine torovirus isolate that replicates in cell culture; since most molecular data have been obtained with this isolate, BEV has been acknowledged as the torovirus prototype.

Recognition of toroviruses as a new group of potentially pathogenic viruses came 7 years after the discovery of BEV, when morphologically similar particles were discovered by electron microscopy (EM) in stool specimens from calves with severe diarrhea in a dairy herd in Breda, Iowa (Fig. 1B) (Woode *et al.*, 1982). Two further strains of Breda virus (BRV) have been reported; one was detected in feces from a 5-month-old diarrheal calf in Ohio, and a second Iowa strain was recovered from a 2-day-old experimental animal (Saif *et al.*, 1981; Woode *et al.*, 1985). Despite repeated attempts, BRV has not been adapted to growth in cell or tissue culture, a problem which has hampered its biochemical, biophysical, and molecular characterization (Woode, 1987). However, its pathogenesis and pathology have been studied in experimentally infected gnotobiotic calves (Fagerland *et al.*, 1986; Pohlenz *et al.*, 1984; Woode *et al.*, 1982, 1985), showing that BRV infections may cause gastroenteritis.

Recently, Vanopdenbosch *et al.* (1992, 1992b) reported the isolation of a torovirus-like virus from the respiratory tract of calves with pneumonia, suggesting that both enterotropic and pneumotropic bovine toroviruses exist. The authors have named this isolate bovine respiratory torovirus (BRTV); it remains to be shown whether the differences between BRTV and BRV (and between BEV and the bovine isolates) are large enough to justify this nomenclatorial distinction.

Besides the established toroviruses of horses and cattle, torovirus-like particles (TVLPs) have been found by EM in different animal species; torovirus antibodies appear to be widespread in higher vertebrates, indicating that these viruses infect a broad range of animal hosts (Muir *et al.*, 1990; Scott *et al.*, 1987; Weiss *et al.*, 1984). The



TABLE I  
TAXONOMIC RELATIONSHIPS BETWEEN CORONA- AND TOROVIRUSES

| Family        | Genus                         | Antigenic group    | Name   | Reference <sup>a</sup>                                   |                            |
|---------------|-------------------------------|--------------------|--|--|----------------------------|
| Coronaviridae | <i>Torovirus</i> <sup>a</sup> |                    | Berne virus; equine torovirus (BEV)              | Weiss <i>et al.</i> (1983)                               |                            |
|               |                               |                    | Breda virus 1/2; bovine enteric torovirus (BRV)  | Saif <i>et al.</i> (1981),<br>Woode <i>et al.</i> (1982) |                            |
|               |                               |                    | Bovine respiratory torovirus (BRTV) <sup>b</sup> | Vanopdenbosch <i>et al.</i> (1991)                       |                            |
|               |                               |                    | Porcine torovirus (PTV) <sup>b</sup>             | Scott <i>et al.</i> (1987)                               |                            |
|               |                               |                    | Feline torovirus (FTV) <sup>b</sup>              | Muir <i>et al.</i> (1990)                                |                            |
|               |                               |                    | Human torovirus (HTV)                            | Beards <i>et al.</i> (1984)                              |                            |
|               |                               | <i>Coronavirus</i> | I  |  | Feline enteric coronavirus |
|               |                               |                    |  | Feline infectious peritonitis virus                      |                            |
|               |                               |                    |  | Canine coronavirus                                       |                            |
|               | II                            |                    |  | Transmissible gastroenteritis virus                      |                            |
|               |                               |                    |  | Human coronavirus 229E                                   |                            |
|               |                               |                    |  | Bovine coronavirus <sup>c</sup>                          |                            |
|               | III                           |                    |  | Human coronavirus OC43                                   |                            |
|               |                               |                    |  | Hemagglutinating encephalitis virus                      |                            |
|               |                               |                    | Infectious bronchitis virus                      |  |                            |

<sup>a</sup>Pringle (1992).

<sup>b</sup>Not confirmed by others.

<sup>c</sup>Only the coronaviruses of animal species that are infected by toroviruses are listed, with the exception of infectious bronchitis virus.

<sup>d</sup>For the toroviruses the first description is given as a reference. Members of the genus *Coronavirus* and their relationships are listed as reviewed by Wege *et al.* (1982).

possibility of a torovirus infecting humans was first reported in 1984 (Beards *et al.*, 1984) (Fig. 1C) and has become more likely in view of our own recent data. This review is intended to update information about toroviruses and to describe similarities and differences with the related coronaviruses.



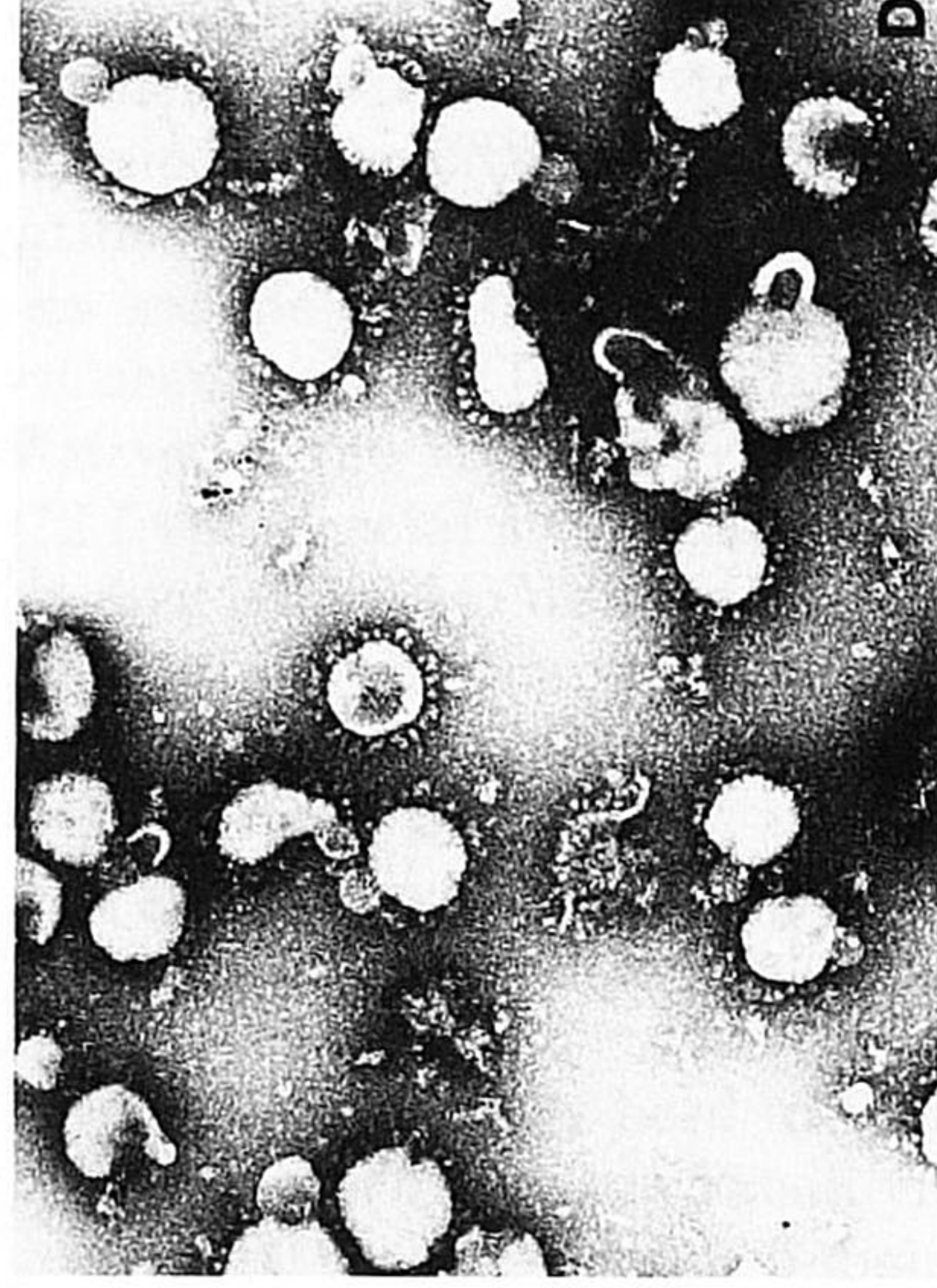
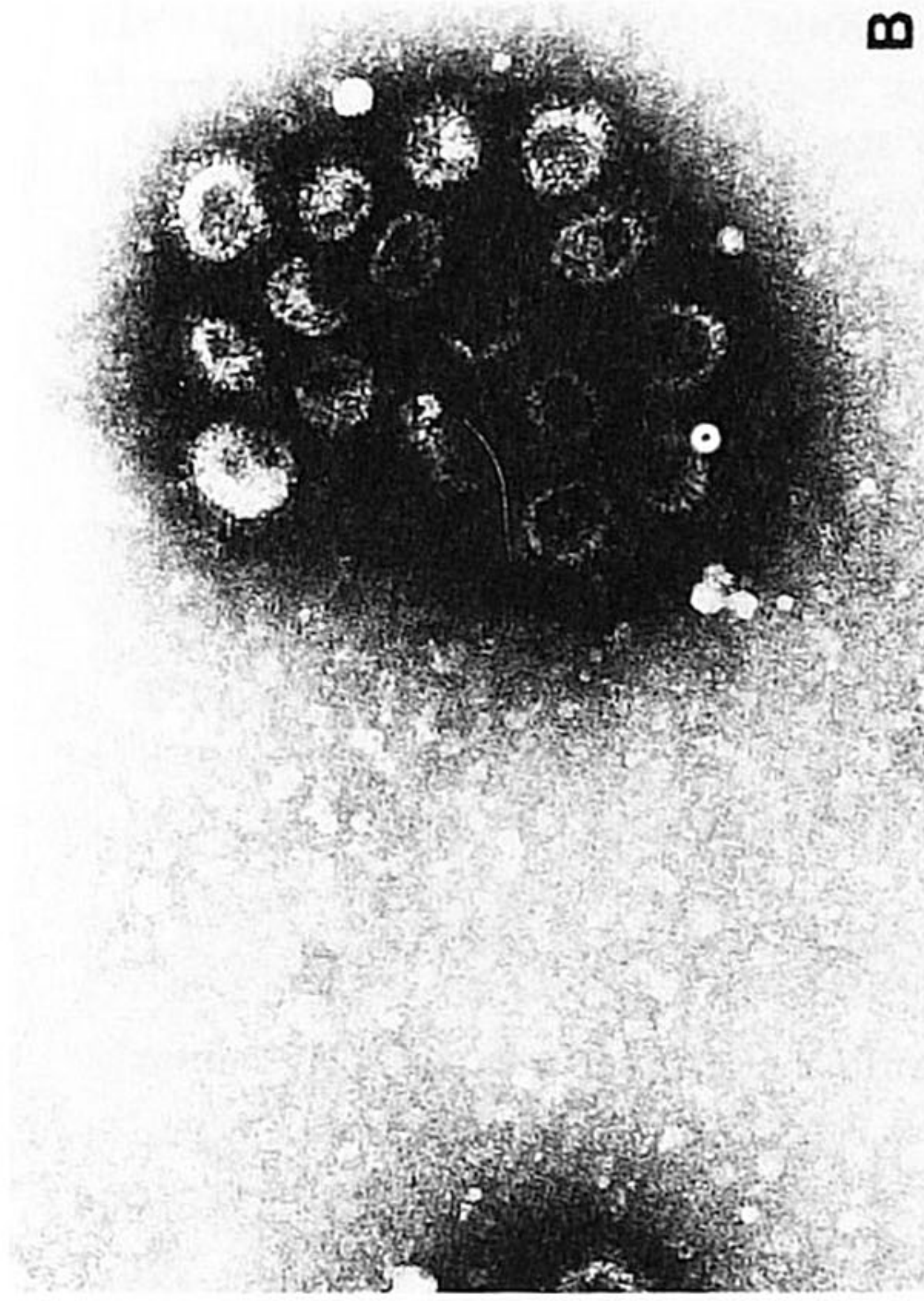
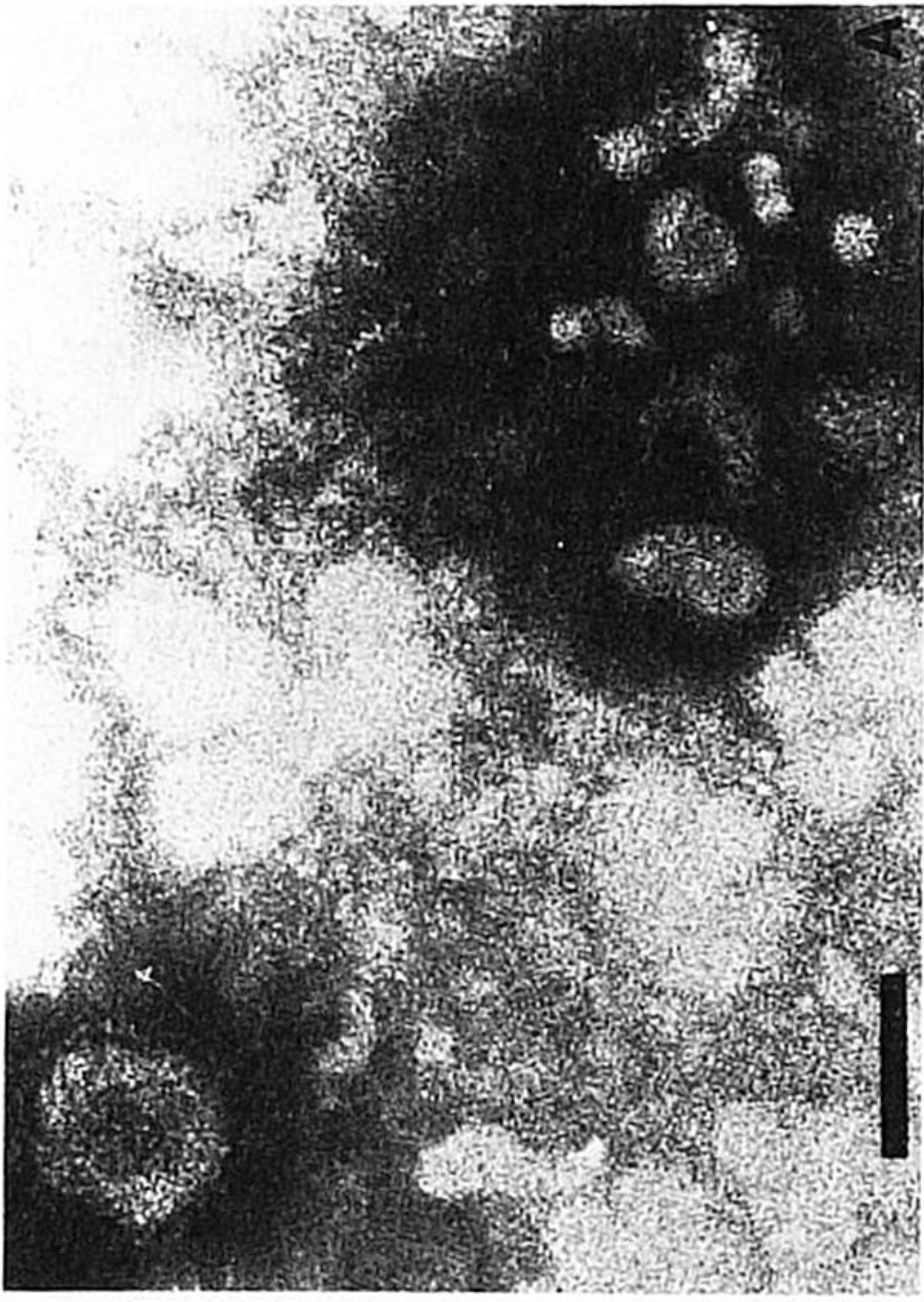


FIG. 1. Electron micrographs showing (A) Berne virus in two orientations, revealing its erythrocyte-like shape; (B) Breda virus; (C) torovirus-like particles in human stool specimens; and (D) human coronavirus 229E, after negative staining. Bar, 100 nm.



## II. VIRUSES

### A. *Biological Characteristics*

This chapter mostly contains information on BEV, which, until recently, was the only tissue culture-adapted torovirus. Any available information for the other toroviruses is discussed at the end of each section.

#### 1. *Isolation and Growth in Culture*

BEV can be propagated in equine dermis or embryonic mule skin (EMS) cells, in which it causes a cytopathic effect that results in cell lysis (Weiss *et al.*, 1983). Trypsin or  $\beta$ -chymotrypsin treatment of seed virus prior to tissue culture inoculation gave a marked increase in infectivity (Weiss and Horzinek, 1986a), but the addition of trypsin to the culture medium is not essential, unlike the situation with other enterotropic viruses. Repeated attempts at the Berne Veterinary Faculty to isolate additional torovirus strains from horses have been unsuccessful, suggesting that BEV is a mutant virus that was modified in a way enabling it to replicate in cell culture.

Isolation attempts for bovine enteric toroviruses have been less successful, in spite of intensive and repeated attempts made with BRV. Woode *et al.* (1982) studied the Madin–Darby bovine kidney cell line as well as organ cultures of tracheal and intestinal tissue; they found no evidence of BRV replication by immunofluorescence (IFA) and hemagglutination (HA) assay, under conditions in which bovine coronavirus multiplied. In contrast, Vanopdenbosch *et al.* (1992b) recently reported the isolation of BRTV in Madin–Darby bovine kidney cells for a few initial passages, followed by cultivation in kidney cells from a wide range of animal species. Infection was detected by scoring for cytopathic effect, and the results were confirmed by IFA and EM. Only a few passages could be done on the same cell type, an effect the authors attribute to the production of interferon (Vanopdenbosch *et al.*, 1992b). These results still need to be confirmed.

No toroviruses have been isolated from humans, cats, or pigs; only physical evidence of the presence of toroviruses in these hosts has been obtained. For the sake of brevity, these particles are referred to as human (HTV), feline (FTV), and porcine toroviruses in this review. The particles coexisting with parvovirions in a case of canine enteritis (Hill and Yang, 1984)—enveloped, 110 nm in diameter, with kidney-shaped cores—have not been further characterized.



## 2. *Host and Host Cell Range*

In culture, replication of BEV is restricted to cells of equine origin (Weiss and Horzinek, 1987). The host range and *in vivo* tissue tropisms of BEV have not been studied.

In BRV-infected cattle, viral antigen can be found in the dome epithelium and in enterocytes of the lower small intestine and the large intestine (Pohlenz *et al.*, 1984; Woode *et al.*, 1984). No evidence of viral infection was obtained in inoculated rats, mice, or lambs (Woode *et al.*, 1982; Woode, 1987).

BRTV reportedly grows in cells of bovine, canine, feline, and simian origin (Vanopdenbosch *et al.*, 1992b). In BRTV-infected calves, IFA-positive foci were seen in the larynx, trachea, and lungs when convalescent-phase sera from experimentally BRV-infected calves were used (Vanopdenbosch *et al.*, 1992b). Infection experiments on animals other than cattle have not been done.

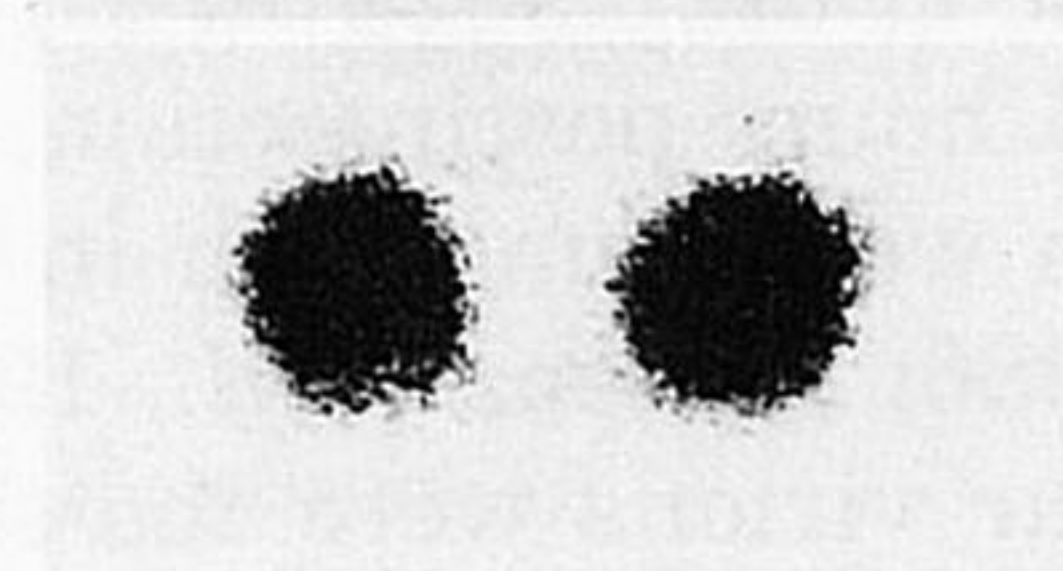
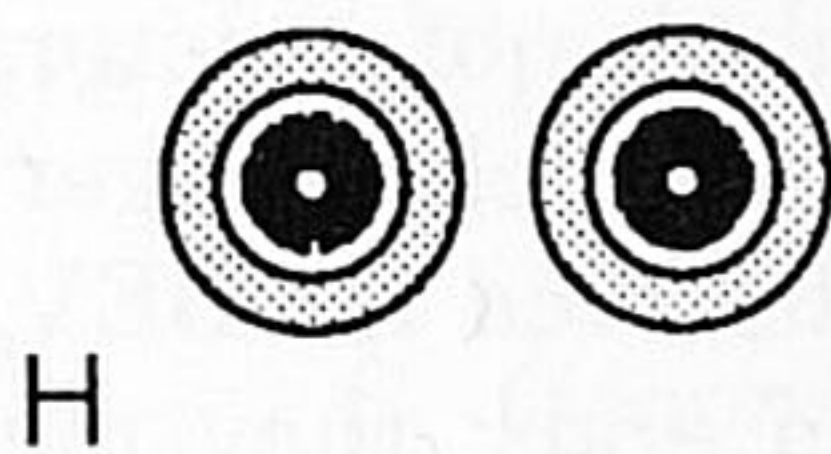
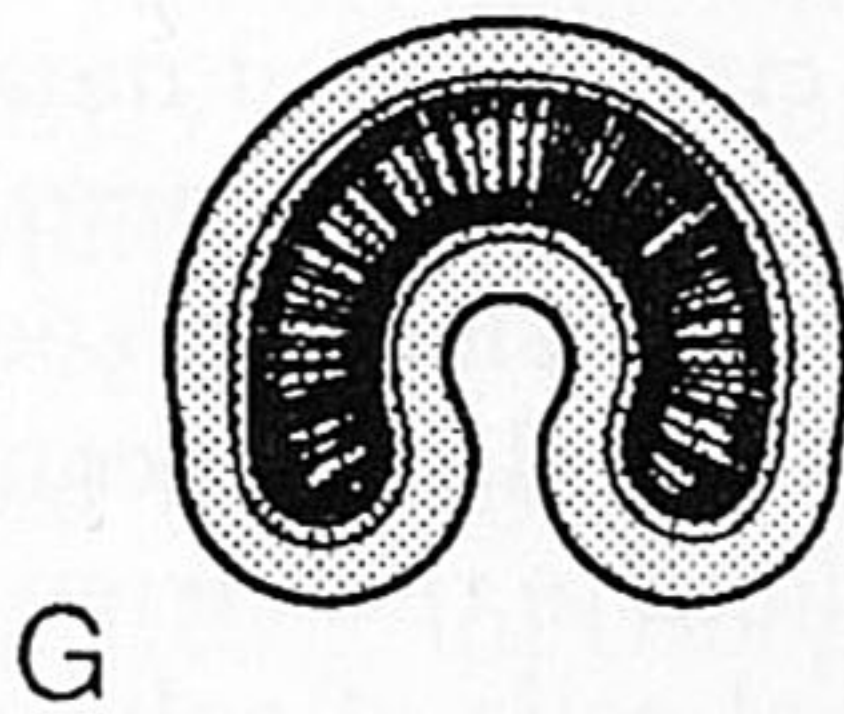
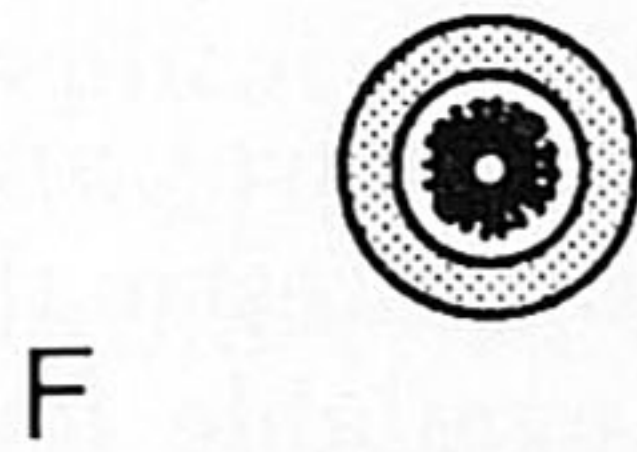
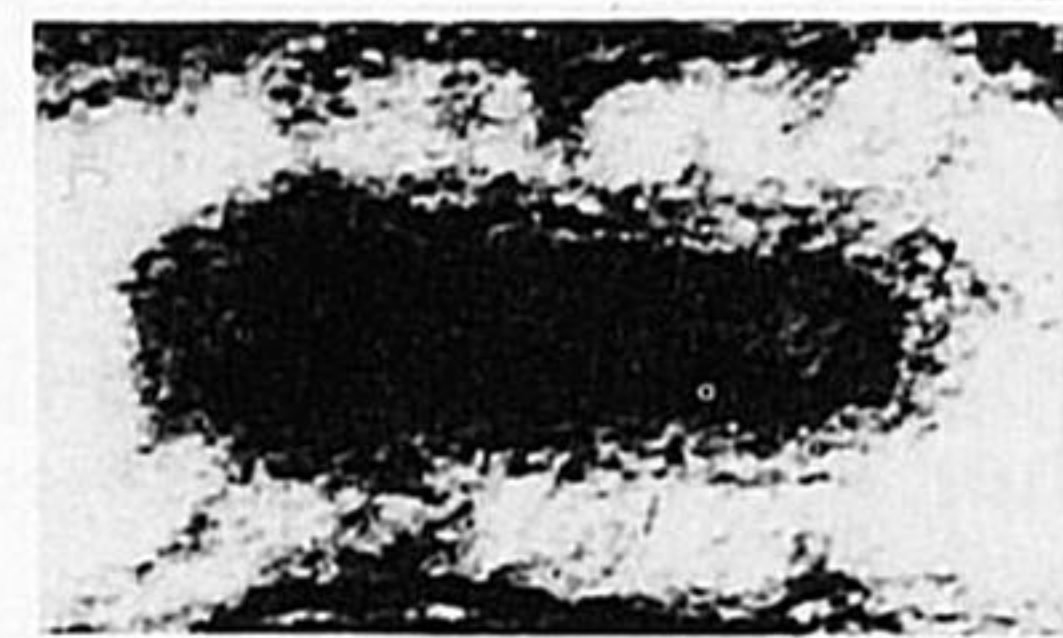
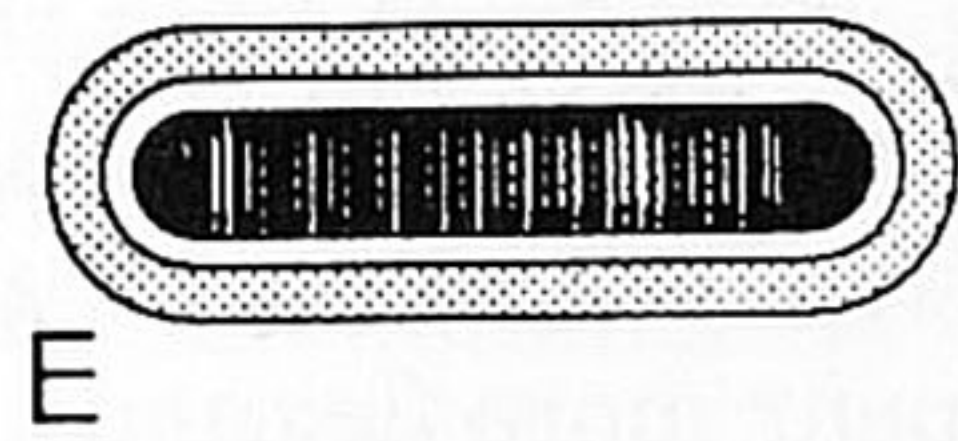
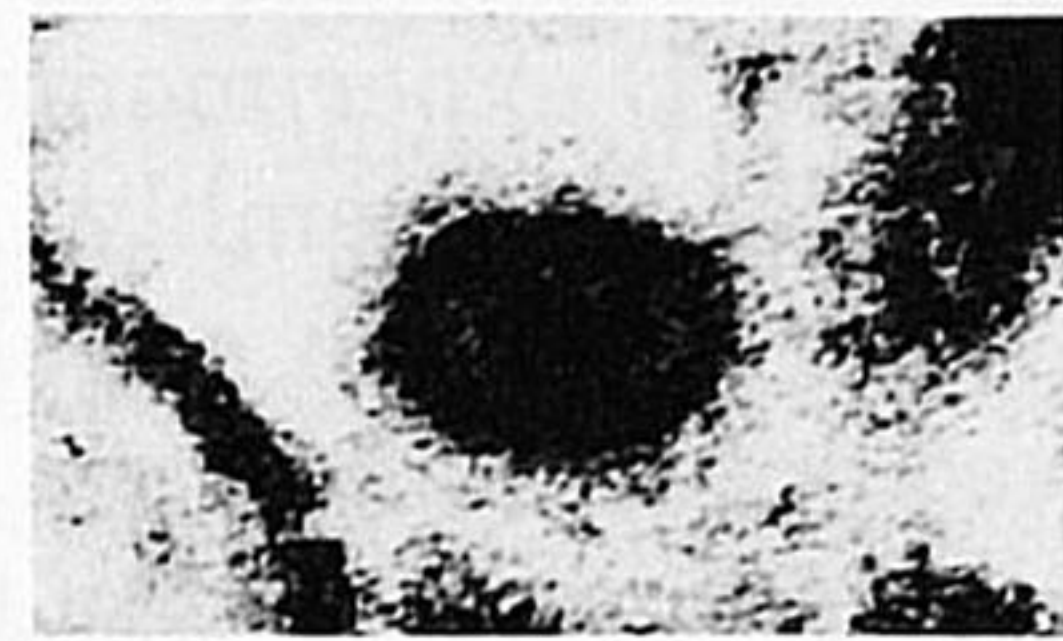
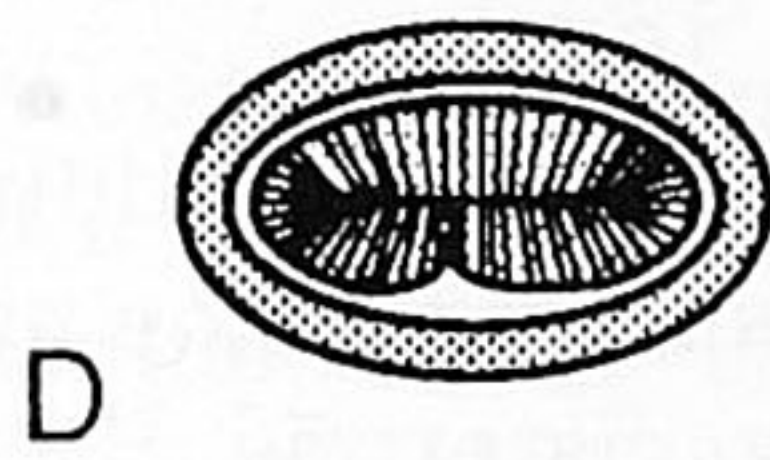
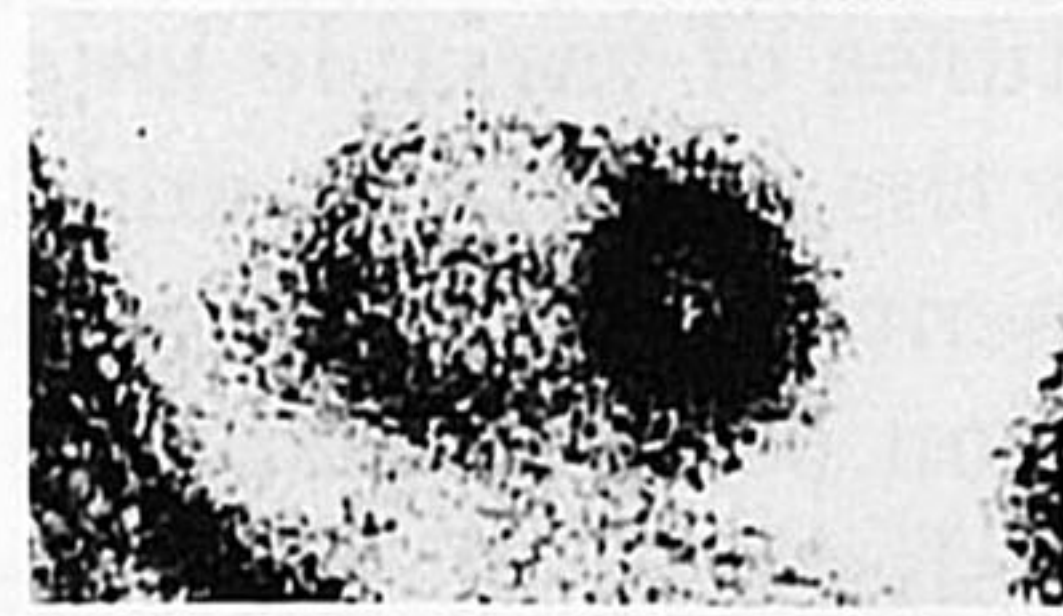
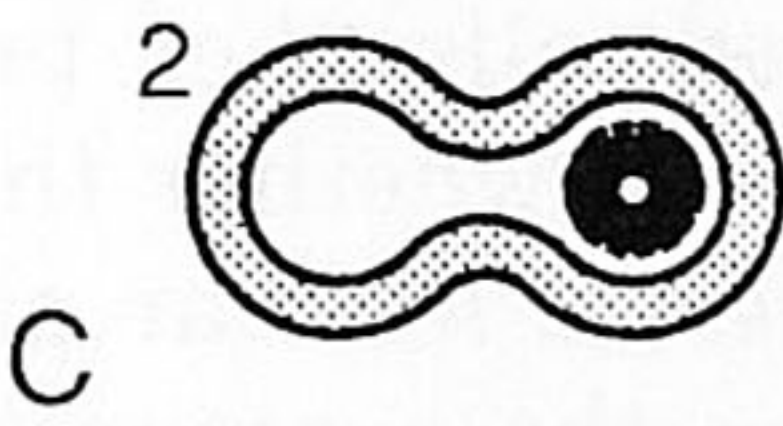
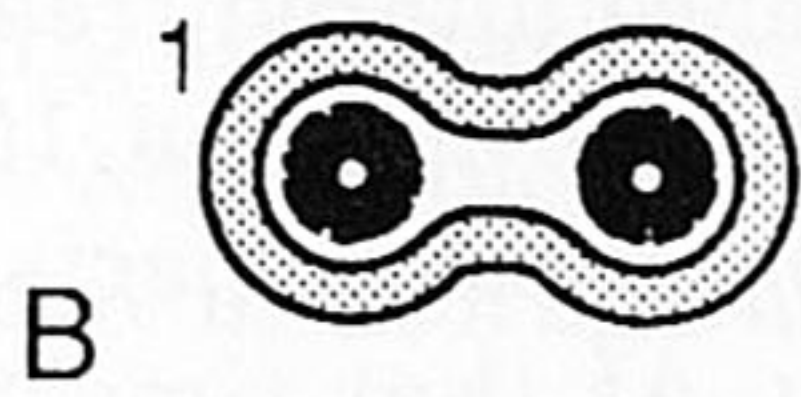
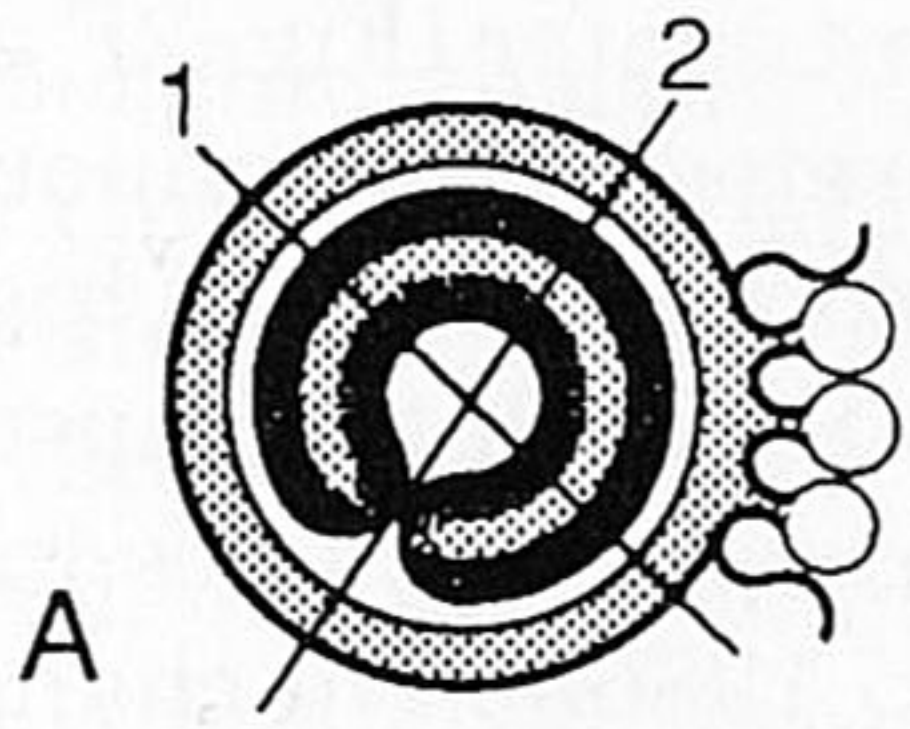
## 3. *Morphology and Morphogenesis*

The morphological features of BEV have been studied in detail with cell culture adapted virus in equine dermis cells. Extracellular BEV particles contain a helical nucleocapsid that is coiled into a hollow tube (diameter, 23 nm; average length, 104 nm; periodicity, 4.5 nm); the nucleocapsid is straight (Fig. 2E), is bent into a C shape (Fig. 2G), or appears as an open ring (Fig. 2A). This structure is surrounded by a tightly adherent envelope about 11 nm thick. Consequently, the virion assumes an erythrocyte-like or kidney shape, depending on whether the membrane bridges the gap between the adjacent ends of the nucleocapsid (Fig. 2A–D) or follows its small curvature (Fig. 2F–H). The largest diameter of BEV is estimated at 120–140 nm. Club-shaped projections (average length, 20 nm), which are referred to as peplomers in analogy to coronaviruses, are present on the virion surface (Figs. 1A and 2A) (Weiss *et al.*, 1983). In thin sections through infected cells, most particles appear rod-like (Fig. 2E); transversal sections show

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FIG. 2. Different forms of BEV particles seen in ultrathin sections through BEV-infected equine dermis cells. On the right are electron micrographs of BEV particles; on the left, schematic interpretations of the viral structures seen in the corresponding photographs. (A) Virion with a toroidal core within a circular particle outline. The indicated section plane 1 leads to a biconcave structure with twin circular cross-sections of the core (B), section plane 2 cuts the nucleocapsid only once (C); (D) elliptical virion with little resolution of the interior; (E) rod-shaped particle; (F) circular structure with an electron-lucent center corresponding to a cross-section through a rod-shaped particle; (G) virion with a C-shaped nucleocapsid; and (H) cross-section through G cutting the nucleocapsid twice. (From Weiss and Horzinek, 1986.)







three concentric circles with an electron-lucent center (Fig. 2F and 2H) (Weiss and Horzinek, 1986b). They are interpreted as the tenuous outer two leaflets of the unit membrane, with the compact ring of high electron density resulting from the cross section through the capsid tube.

The morphogenesis of BEV has been deduced from studies in infected equine dermis cells (Weiss and Horzinek, 1986b). No changes are visible by EM until 6 hours postinfection. Between 6 and 9 hours postinfection large polysomal aggregates are encountered free in the cytoplasm, and viral proteins can be detected by IFA. About 10 hours postinfection virus particles are first seen both within parts of the unaltered Golgi apparatus and extracellularly. At that time tubular structures of variable length, diameter, and electron density are visible in the cytoplasm and the nucleus of infected cells; they probably represent preformed nucleocapsids. It is unknown whether the accumulation of nucleocapsids in the nucleus reflects a nuclear phase in the replication of BEV [as was suggested from the  $\alpha$ -amanitin and actinomycin D experiments described below (Horzinek *et al.*, 1984)] or aberrant virion assembly (Weiss and Horzinek, 1986b).

BEV particles are enveloped by a budding process. Budding was observed predominantly in the Golgi system, resulting in the presence of virions accumulating in the lumen of the Golgi cisternae. Preformed nucleocapsid tubules approach the Golgi membrane with one of the rounded ends, followed by attachment along one side. During the budding process the BEV nucleocapsid is apparently stabilized, leading to a higher electron density and a constant diameter [23 nm (Weiss and Horzinek, 1986b)]; nonenveloped capsids appear more flexuous, with a ragged outline.

In contrast to the situation for BEV, the morphological and morphogenetic features of BRV had to be studied using intestinal tissue from infected calves; hence, less information is available for these viruses. Negatively stained BRV virions appear either kidney-shaped and measuring 30–120 nm, or approximately circular and measuring 75–90 nm. Their envelope bears peplomers 8–10 nm in length (Fig. 1B) (Woode *et al.*, 1982). Longer peplomers are occasionally seen (17–24 nm), especially on virions of the Ohio strain and the second Iowa strain (Woode *et al.*, 1985), but they may not belong to the virion proper (Woode *et al.*, 1982). In virus-infected intestinal cells of calves killed 48–96 hours postinfection, 21-nm-diameter tubules of indeterminate length were found both in the cytoplasm and in nuclei (Fagerland *et al.*, 1986; Pohlenz *et al.*, 1984). As has been observed for BEV, intracellular virions were rod-shaped with rounded ends; they measured 35–40 nm in diameter and were 80–100 nm long (Fagerland *et al.*, 1986; Pohlenz *et al.*, 1984).



Pleomorphic virus-like particles have been observed by EM in the feces of children and adults with diarrhea in France, Great Britain, The Netherlands, and Canada (Beards *et al.*, 1984; Horzinek and Weiss, 1984a; Koopmans *et al.*, 1993b). The particles were spherical, elongated, or kidney-shaped, ranged in diameter from 100 to 150 nm, and had a fringe of closely spaced peplomers 7–9 nm long and a toroidal nucleocapsid-like structure with a diameter of 24 nm (Fig. 1C). According to Beards *et al.* (1986), the internal toroidal structure is hardly ever visible when freshly prepared samples are examined, but appears after storage of the grids for a few days.

Detailed morphological descriptions of BRTV and FTV have not yet been given.

### *B. Physical and Chemical Properties*

The presence of essential lipids in the BEV virion was shown by the complete loss of infectivity after treatment with chloroform or diethyl ether (Weiss and Horzinek, 1986a). In sucrose gradients the virus bands at a density of 1.16 g/ml, which is also indicative of the presence of an envelope (Weiss *et al.*, 1983). The lipids are not readily accessible, however, since viral infectivity is hardly influenced by treatment with phospholipase C or sodium deoxycholate (Weiss and Horzinek, 1986a). BEV is not inactivated at pH values between 2.5 and 10 nor by trypsin or chymotrypsin treatment. On the contrary, enzyme treatment resulted in an enhancement of infectivity [5- to 10-fold (Weiss and Horzinek, 1986a)].

The replication of BEV is inhibited if actinomycin D, an inhibitor of DNA transcription, is added to the culture medium during the first 8 hours postinfection.  $\alpha$ -Amanitin, which specifically inhibits the DNA-dependent RNA polymerase II, has a similar effect; this observation supports the assumption that cellular gene expression has some function in BEV growth (Horzinek *et al.*, 1984).

For BRV the reported buoyant densities in sucrose range from 1.14 (Beards *et al.*, 1986) to 1.18 g/ml (Koopmans *et al.*, 1986). A poorly preserved BRV1 preparation showed heterogeneous banding between 1.18 and 1.21 g/ml (Koopmans *et al.*, 1986). Toroviruses prepared from human feces band at the same density as BRV (Beards *et al.*, 1986; Koopmans *et al.*, 1993b).

### *C. Genome Organization and Replication*

The growth of BEV is unaffected by 5'-iodo-2'-deoxyuridine, which gave the first indication for the presence of an RNA genome (Weiss *et*



*al.*, 1983). The RNA molecule is approximately 25–30 kb long, is single stranded, and can be isolated using oligo(dT) affinity chromatography, indicating that it is polyadenylated (Weiss and Horzinek, 1987; Snijder *et al.*, 1988). Its positive polarity was evidenced by transfection of EMS cells, leading to a cytopathic effect and the production of infectious virus (Horzinek *et al.*, 1987; Snijder *et al.*, 1988). In EMS cells BEV directs the synthesis of five virus-specific polyadenylated RNA species with estimated lengths of 0.8, 1.4, 2.1, 7.5, and 25–30 kb, which form a nested set; the sequence of each RNA is contained within the sequences of all larger RNAs, and their 3' ends are identical (Fig. 3) (Snijder *et al.*, 1988, 1990a). This expression strategy places toroviruses in a cluster along with caliciviruses (Black *et al.*, 1978), coronaviruses (Spaan *et al.*, 1981; Siddell *et al.*, 1983), and arteriviruses [equine arteritis virus, lactate dehydrogenase-elevating virus, simian hemorrhagic fever virus, and the Lelystad/porcine reproductive and respiratory syndrome virus (Plagemann and Moennig, 1992; Wensvoort *et al.*, 1991)].

About 15 kb of the BEV genome has been sequenced, and six open reading frames (ORFs) have been identified (ORFs 1a, 1b, 2, 3, 4, and 5; Fig. 3). The initiation codons of ORFs 2–5 are located near the 5' ends of RNA 2–5, and there is no common leader sequence, unlike the situation in the subgenomic RNAs of coronaviruses. The 5' end of ORF 1a remains to be sequenced (Snijder *et al.*, 1989, 1990a–c).

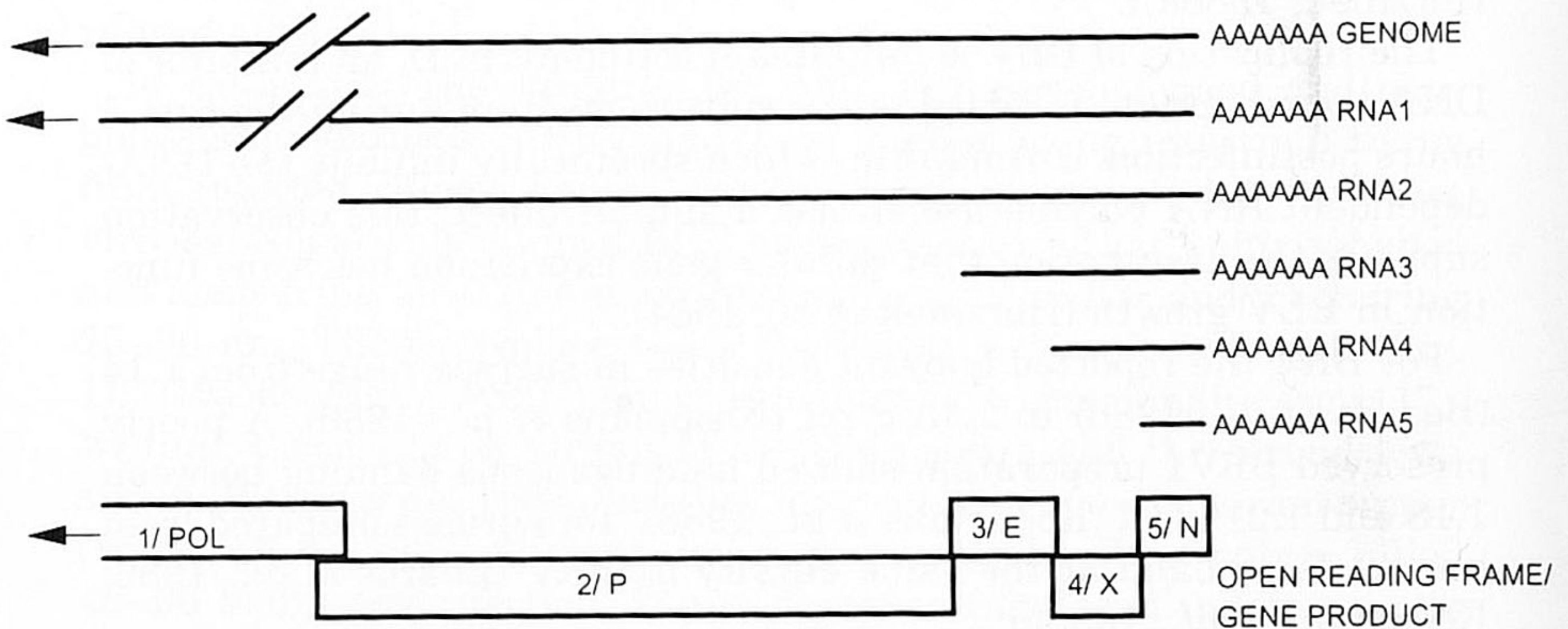


FIG. 3. Genome, subgenomic mRNAs (RNA1–5), and genomic organization (open reading frames) of torovirus. The numbers of the open reading frames correspond with the RNA numbers (e.g., open reading frame 1 is expressed from RNA1). The gene products are the polymerase protein (POL), the peplomer proteins (P), the envelope protein (E), an unidentified potential product (X), and the nucleocapsid protein (N).



### D. Protein Composition and Antigenic Determinants

The structural proteins of BEV were first identified by metabolic labeling experiments; polypeptides of 20, 22, 37, and 80–100 kDa copurified with infectious virions and were immunoprecipitated using sera from immunized rabbits (Fig. 4, left, lane 3) (Horzinek *et al.*, 1984). On detergent treatment of the virions, the 22-, 37-, and 80- to 100-kDa species were converted into slowly sedimenting material, which indicated their membrane association (Horzinek *et al.*, 1985). Only the 20-kDa protein is present in purified BEV nucleocapsids; it was accordingly named the nucleocapsid (N) protein. It is phosphory-

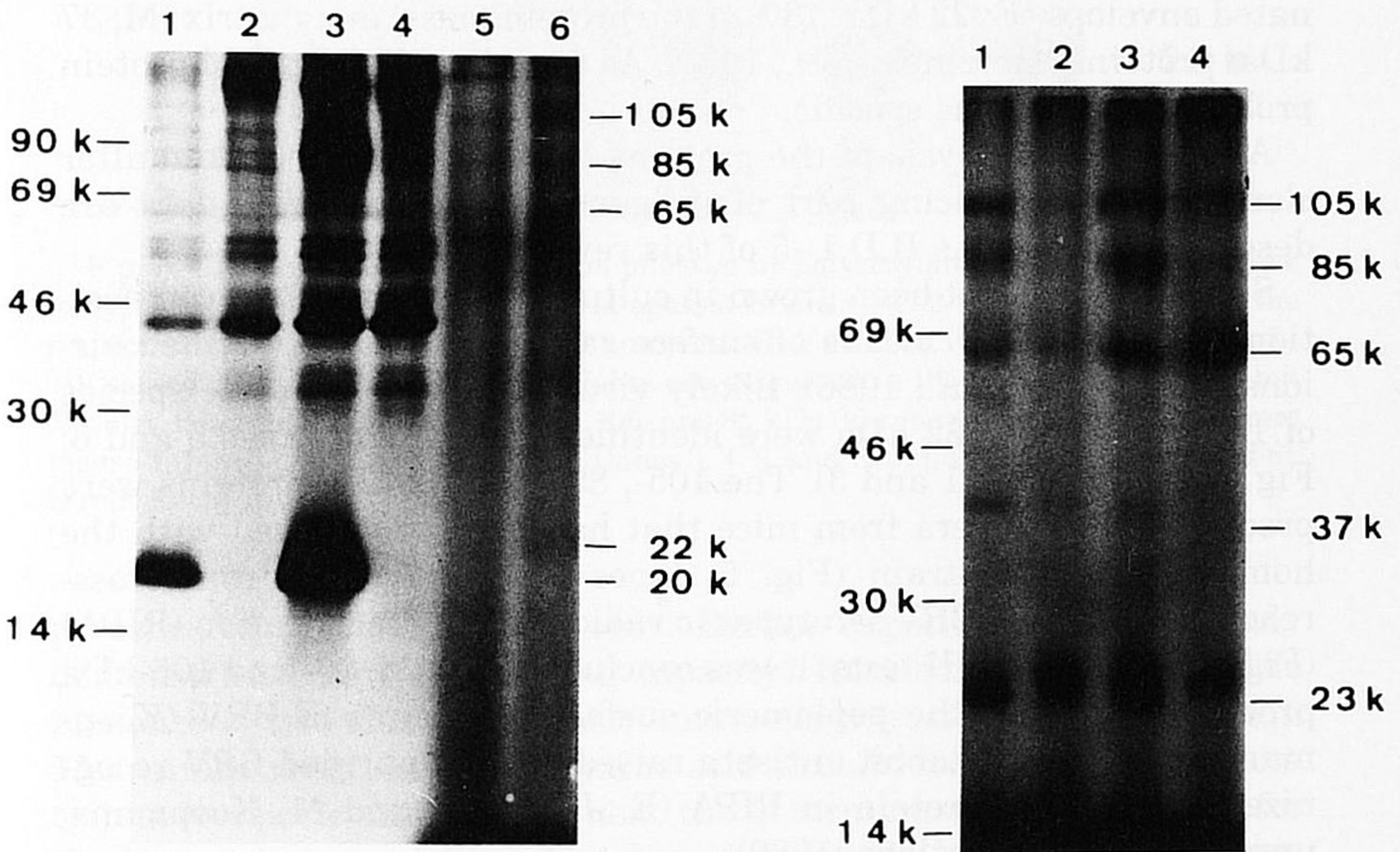


FIG. 4. (Left) [ $^{35}\text{S}$ ]Methionine-labeled Berne virus proteins from infected (lanes 1 and 3) and noninfected (lanes 2 and 4) embryonic mule skin cells after radioimmune precipitation was done with a homologous rabbit hyperimmune serum and *Staphylococcus aureus* protein A (lanes 3 and 4) and protein A alone (lanes 1 and 2). In lanes 5 and 6  $^{125}\text{I}$ -labeled preparations of Breda virus 1 (lane 5) and Breda virus 2 (lane 6) were loaded. The numbers to the left of the fluorographs indicate the molecular weights of the following  $^{14}\text{C}$  marker proteins coelectrophoresed in the same gel: phosphorylase (90 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), and lysozyme (14 kDa). The calculated molecular weights [in kilodaltons (k)] of the viral proteins are indicated to the right of the figure. (Right) Polyacrylamide gel electrophoresis analysis of radioiodinated preparations of purified Breda virus 1 (lanes 1 and 2) and Breda virus 2 (lanes 3 and 4) before (lanes 1 and 3) and after (lanes 2 and 4) extraction with diethyl ether was done. (From Koopmans *et al.*, 1986.)



lated, binds RNA, and accounts for 84% of the protein mass of the virion (Horzinek *et al.*, 1985). The heterogeneous 80- to 100-kDa material (1% of the protein mass; Fig. 4, left, lane 3) is recognized by both neutralizing and hemagglutination-inhibiting (HI) monoclonal antibodies and was therefore postulated to represent the peplomer (P) protein (Horzinek *et al.*, 1986; Kaeffer *et al.*, 1989). BEV hemagglutinates human blood group O, rabbit, and guinea pig erythrocytes; transmission EM showed that virus particles form bridges between adjacent erythrocytes (Zanoni *et al.*, 1986). Antipeplomer monoclonal antibodies neutralize BEV infectivity and inhibit HA, showing that the hemagglutinin is associated with the P proteins (Kaeffer *et al.*, 1989).

The two remaining membrane-associated polypeptides were designated envelope (E; 22 kDa, 13% of the protein mass) and matrix (M; 37 kDa) proteins (Horzinek *et al.*, 1986). As explained later, the M protein probably is not virus specific.

A molecular analysis of the proteins of BEV was undertaken after cloning and sequencing part of its genome. The resulting data are described in Sections II,D,1-5 of this review.

Since BRV has not been grown in cultured cells, its protein composition was studied by means of surface radioiodination of purified virions (Koopmans *et al.*, 1986). Likely virus-specific polypeptide species of 105, 85, 37, and 22 kDa were identified (Fig. 4, left, lanes 5 and 6; Fig. 4, right, lanes 1 and 3). The 105-, 85-, and 22-kDa proteins were precipitated with sera from mice that had been immunized with the homologous BRV strain (Fig. 5, lanes 6, 8, and 10). From cross-reactivity between BRV serotypes in radioimmunoprecipitation (RIPA) (Fig. 5, lane 2) and HI tests, it was concluded that the 85- and 105-kDa proteins represent the peplomeric surface structures of BRV (Koopmans *et al.*, 1986). Rabbit antisera raised against purified BRV recognized the BEV P protein in RIPA (E. J. Snijder and M. Koopmans, unpublished observations, 1989).

### 1. The N Protein of BEV

The smallest subgenomic RNA (RNA5, 0.8 kb; Fig. 3) contains the first ORF upstream of the poly(A) tail. From the sequence of this gene, a protein of 160 amino acids was predicted. Its calculated  $M_r$  value of 18,300 approximates the 20 kDa of the N protein as estimated from its migration in polyacrylamide gels. *In vitro* translation of RNA5 and of transcripts from the cloned gene, followed by RIPA, was used to identify the gene. Confirmation was obtained from metabolic labeling with either [ $^{35}$ S]methionine or [ $^{35}$ S]cysteine with the knowledge that cysteine residues do not occur in the amino acid sequence of the N protein (Snijder *et al.*, 1988, 1989); that is, the N protein could be labeled in the



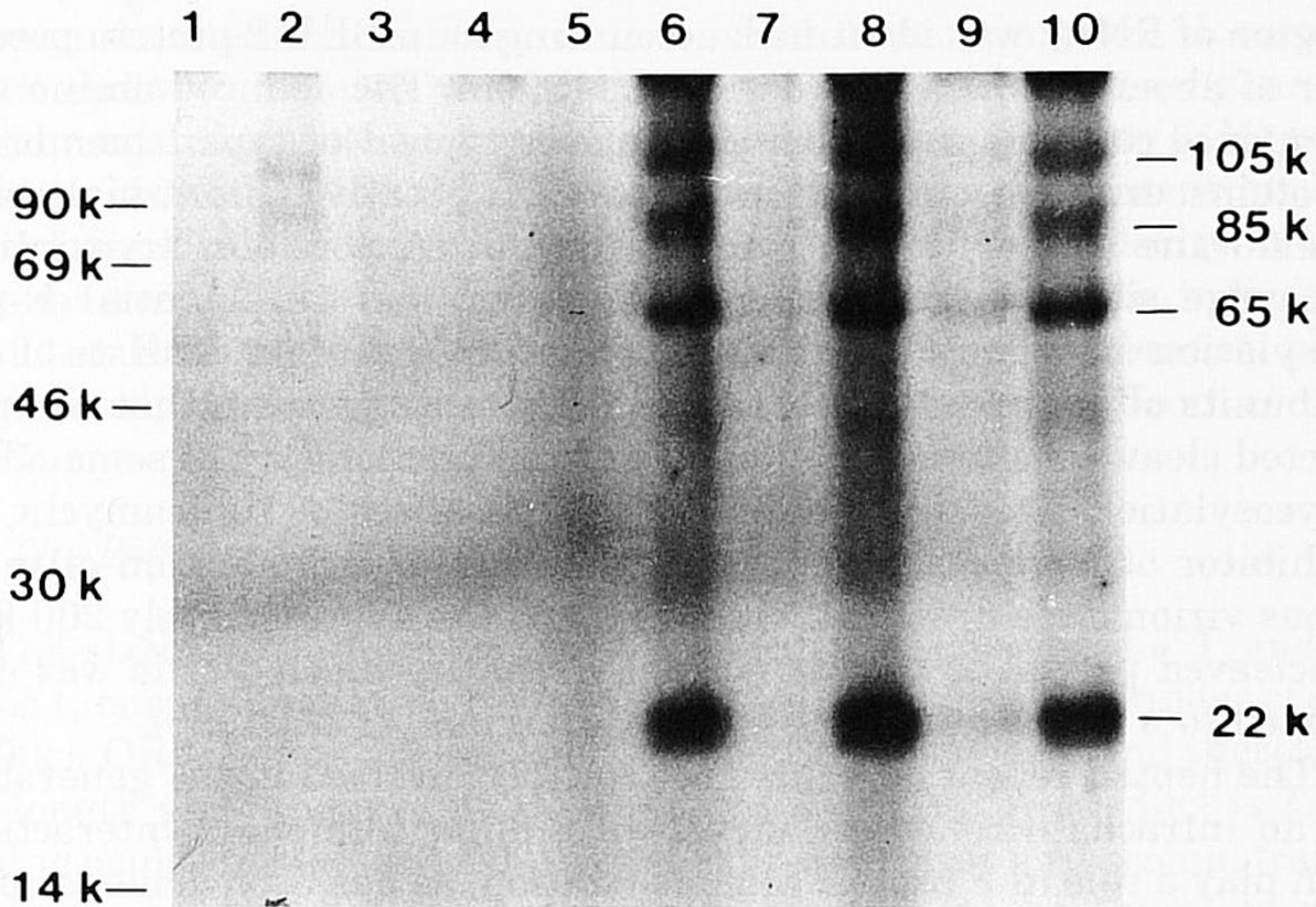


FIG. 5. Radioimmuno precipitation followed by polyacrylamide gel electrophoresis, using three mouse immune sera raised against Breda virus 2 (even numbers) and the respective preimmunization sera (odd numbers); BRV1 (lanes 1–4) and BRV2 (lanes 5–10) served as antigen. Molecular weights are expressed in thousands. Note weak, but distinct, heterologous reactions in the 105- and 85-kDa (k) range (lane 2). Sera were from mouse 1 (lanes 1, 2, 7, and 8), mouse 2 (lanes 3, 4, 9, and 10), and mouse 3 (lanes 5 and 6). (From Koopmans *et al.*, 1986.)

presence of [ $^{35}\text{S}$ ]methionine only. Earlier experiments had already demonstrated its RNA-binding capacity (Horzinek *et al.*, 1985). The presence of two clusters of basic amino acids (residues 34–81 contain 15 arginine residues in a 47-amino-acid stretch; residues 118–156 contain seven basic amino acids near the C terminus) suggests a role for the N protein in nucleic acid binding, an essential step in virion assembly (Snijder *et al.*, 1989).

Smaller N-related polypeptides encountered in BEV-infected cell lysates (Horzinek *et al.*, 1985) were shown to be products of aberrant translation due to initiation on AUG codons further downstream in the N protein gene (Snijder *et al.*, 1989).

## 2. The P Protein

A conspicuous, though not unique, ingredient of torovirion architecture is the surface projection, the club- or petal-shaped peplomer. On the basis of *in vitro* translation studies with BEV, RNA2 was identified as the mRNA for the synthesis of the P protein precursor (Fig. 3) (Snijder *et al.*, 1988). An ORF of 4743 nucleotides in the unique 5'



region of RNA2 was identified, accounting for a BEV P protein precursor of about 178 kDa (Snijder *et al.*, 1990b). The deduced amino acid sequence contains a number of domains typical of type I membrane proteins: an N-terminal signal sequence, a putative C-terminal transmembrane anchor, and a cytoplasmic tail. A possible "trypsinlike" cleavage site, two heptad repeat domains, and 18 potential N-glycosylation sites were identified. The mature P protein consists of two subunits of approximately 111 and 65 kDa, suggesting that the predicted cleavage site is functional *in vivo*. Functionality of some of the glycosylation sites was confirmed by the effect of tunicamycin, an inhibitor of N-glycosylation; in its presence, the production of infectious virions was drastically reduced, and the approximately 200-kDa uncleaved precursor that is normally seen in infected cells was converted to a faster-migrating protein (Horzinek *et al.*, 1986).

The heptad repeat domains are probably involved in the generation of an intrachain coiled-coil structure; similar interchain interactions can play a role in P protein oligomerization, as has been demonstrated for coronaviruses (de Groot *et al.*, 1987). Using a sucrose gradient assay, Snijder *et al.* (1990b) showed that in infected cells the P protein is indeed present as a dimer. The intra- and interchain coiled-coil interactions may stabilize the elongated BEV peplomers (Snijder *et al.*, 1990b).

### 3. The E Protein of BEV

The smallest and most abundant membrane-associated polypeptide of BEV is the E protein; it is unglycosylated (Horzinek *et al.*, 1986) and accounts for about 13% of the virion protein mass (Horzinek *et al.*, 1985). *In vitro* studies have shown that it is translated from the ORF located at the 5' end of RNA3 (Fig. 3) (Snijder *et al.*, 1990a). The nucleotide sequence of the E protein gene of BEV was determined, and its 26.5-kDa translation product was identified by *in vitro* transcription and translation. In polyacrylamide gels the E protein migrates with an apparent mass of a 22-kDa protein, probably because of its strong hydrophobicity (den Boon *et al.*, 1991a). Computer analysis of the protein sequence revealed the characteristics of a class III membrane protein containing three successive transmembrane  $\alpha$ -helices in the N-terminal half, very similar to the coronaviral membrane (M) protein (den Boon *et al.*, 1991a). Proteinase K digestion experiments using E protein that had been expressed in the presence of microsomes showed that only small portions of either end of the polypeptide are exposed on opposite sides of the vesicle membranes. To determine the orientation of the E protein in the membrane, an expression plasmid



was constructed that contained most of the E protein gene. However, its 3' end was replaced with that of a coronavirus M protein gene to which an antipeptide serum was available. This construct (E/M) was used to show that the C terminus of the E protein is present at the cytoplasmic side of the membrane, which is another similarity to the coronavirus M protein. Immunofluorescence experiments indicated that the E/M protein accumulated in intracellular membranes, predominantly those of the endoplasmic reticulum (den Boon *et al.*, 1991a).

#### 4. *The Polymerase Protein of BEV*

The slightly overlapping ORFs 1a and 1b, located at the 5' end of RNA1, encode a putative RNA polymerase (POL, Fig. 3) (Snijder *et al.*, 1990c). ORF 1a has not yet been completely sequenced, and therefore its length and product size are unknown. The ORF 1b product is 2291 amino acids long ( $M_r$  261,000) and is expressed after a ribosomal frame shift during translation. A sequence identical to the actual frame-shifting site in the polymerase genes of infectious bronchitis virus and mouse hepatitis virus is present in BEV ORF 1a just upstream of the termination codon. The frame-shifting supposedly is facilitated by the predicted tertiary RNA structure (a pseudoknot) in the overlapping region of ORFs 1a and 1b, as occurs in coronaviruses (Snijder *et al.*, 1990c).

Similarities in genome expression and amino acid sequence motifs of the polymerase gene between toroviruses and coronaviruses have led to a revision of our original notion that the viruses are unrelated, as discussed in Section VIII.

#### 5. *The "Matrix Protein" of BEV*

This 37-kDa phosphoprotein accounts for about 2% of the virion protein mass; after Triton X-100 treatment it has been found in association with material that sediments more slowly than the virion (Horzinek *et al.*, 1985). It was therefore considered to be membrane associated, but its exact location and origin remain obscure. The M protein is recognized by only a subset of BEV antibody-containing sera (Horzinek *et al.*, 1984, 1985). The available sequence of the BEV genome does not contain an ORF with a coding capacity for a protein in the 37-kDa range. The *in vitro* translation product of ORF 4 ( $M_r$  16,000; Fig. 3) has not been identified in culture supernatants or cell lysates of infected cells (E. J. Snijder and J. A. den Boon, unpublished observations, 1990).



### III. CLINICAL FEATURES

Since BRV has to be propagated in calves and was found to be pathogenic soon after its discovery, whereas BEV still is "a virus in search of a disease," most clinical studies have focused on BRV, making it the main subject here of Sections III and IV. The available information in infection with BRTV, BEV, and other toroviruses is discussed at the end of each section.

#### A. *Experimental Infection*

The three available BRV strains are pathogenic for newborn gnotobiotic and nonimmune conventional calves (Woode *et al.*, 1985). Most of the experimentally infected calves (aged 1 hour to 10 weeks) developed watery diarrhea within 24–72 hours postinfection which lasted 4–5 days, and shed virus for 3–4 days, as determined by HA (Woode *et al.*, 1982, 1983, 1985). The most severe clinical signs occurred 24–48 hours after onset of the diarrhea, and were accompanied by dehydration and weakness (Woode, 1987). In some calves the diarrhea was preceded by a mild temperature reaction (40°C). Diarrhea generally was more severe in the calves with a normal intestinal flora than in gnotobiotic calves. In some infected calves severe depression was observed. One conventional calf developed hyperpnea and a watery eye discharge (Woode *et al.*, 1982, 1985). This finding has become more interesting after the recently described isolations of TVLPs from the respiratory tract of calves with severe pneumonia (Vanopdenbosch *et al.*, 1991, 1992b). Woode *et al.* (1982, 1985) found a reduction of D-xylose resorption in calves, ranging from 15% in animals with mild diarrhea to 65% in severely affected calves. This finding indicates some loss of absorptive capacity of the small intestine, but it is not as extreme as in rotavirus infections (the most common cause of diarrhea in calves), in which there is a 60–100% reduction in the rate of D-xylose absorption. The discrepancy can be explained by differences in cell tropism: Toroviruses mainly infect differentiating epithelial cells in the crypts of the intestinal villi, especially in the large intestine (Woode *et al.*, 1982), whereas rotaviruses predominantly target the mature epithelial (absorptive) cells of the small intestine (Mebus *et al.*, 1971).

BEV had been isolated from a horse with pseudomembranous enteritis and miliary granulomas in the liver, suggesting systemic infection. However, when two yearlings were infected intravenously with  $10^7$  TCID<sub>50</sub> of tissue culture-grown BEV, they seroconverted without accompanying clinical symptoms (Weiss *et al.*, 1984). A 3-day-old



gnotobiotic foal was infected orally with tissue culture-adapted BEV, and again no symptoms were seen, while virus could be recovered from stools and nasal swabs. Attempts to infect a 3-month-old foal were unsuccessful (F. Scott, Moredun Research Institute, Edinburgh, Scotland, personal communication, 1992).

### *B. Natural Infection*

The natural course of infection was studied in sentinel dairy calves that were kept under conventional conditions, except for being physically separated from other cattle until 10 months of age. The healthy colostrum-fed calves had been obtained from different farms and housed together at 1 week of age. Torovirus-associated diarrhea developed in nine of the 10 calves within 3 weeks after their arrival at the experimental facility. The diarrhea lasted between 2 and 13 days, and led to mild dehydration in four of the calves and to signs of general illness in two. None of the calves required therapeutic intervention other than dietary changes and oral rehydration therapy (Koopmans *et al.*, 1990). In a longitudinal study on farms in The Netherlands, torovirus-associated diarrhea was found in slightly older calves than rota- or coronavirus-associated diarrhea (average, 12.7, 7.7, and 8.3 days, respectively); also, torovirus-associated diarrhea lasted longer (average, 9.2, 6.8, and 6.8 days, respectively). Otherwise, the symptoms were similar for all three infections (Koopmans *et al.*, 1991c). Older calves had very mild diarrhea or none at all, in association with torovirus shedding (Koopmans *et al.*, 1991c).

The role of toroviruses in other disease pictures is only emerging, and epidemiological studies are needed to establish the causal relationship between virus presence and disease. Vanopdenbosch *et al.* (1991, 1992b) listed clinical data from 12 calves that were diagnosed positive for BRTV by postmortem examinations of the lungs; 10 had symptoms of respiratory tract infection before they died, and two had diarrhea. The same authors reported evidence of toroviral antigen (as detected by IFA on frozen tissue sections) in 3.2% ( $n = 1723$ ) of the respiratory samples examined, 5.1% ( $n = 213$ ) of placental cotyledons after abortion late in gestation, and 4% ( $n = 3104$ ) of the intestinal tract samples. Respiratory torovirus infections occur mainly during the first month of life and between 4 and 6 months of age, with an autumn peak. In about 25% of all cases, sudden death had occurred; besides pneumonia, tracheitis, and diarrhea, central nervous symptoms have incidentally been observed (Vanopdenbosch *et al.*, 1992a). These data indicate that in cattle, generalized infections must not be excluded.



BEV appears to be of little pathogenic importance, and so far no clinical disease has been associated with infection. In a longitudinal study aimed at monitoring young horses for infection with BEV, seroconversions to BEV occurred between 10 and 12 months of age in all 20 yearlings from a stud farm; no overt clinical symptoms were observed in any of the infected animals (Weiss *et al.*, 1984). A possible role of BEV in diarrhea of young foals has not been studied.

In most studies diarrhea is mentioned in the animals that are shedding torovirus particles or TVLPs, but control groups are not available. Scott *et al.* (1987) reported the presence of TVLPs in feces from a 3-week-old piglet with severe enteritis. The sample was negative for other pathogens, but an association between the presence of TVLPs and diarrhea could not be made since no healthy control animals were examined. In addition, rotavirus and a small round virus were detected in other animals in the herd involved in the disease outbreak, a finding which further obscured a possible etiological relationship between the presence of TVLPs and disease. Similarly, Durham *et al.* (1989) found TVLPs by EM in seven of 72 stool samples from piglets with diarrhea.

Muir *et al.* (1990) reported hemagglutinating TVLPs in feces from specified pathogen-free kittens that had been inoculated with fecal filtrates from a cat with third-eyelid prolapse and diarrhea. Rising titers to the particles were found in five of 50 cats with the disease, but a rise in antibody titer against parvovirus simultaneously occurred in eight of 43 cats tested, against coronavirus in seven of 43, and against rotavirus in 10 of 43. The syndrome was not reproduced in the experimentally infected kittens.

In 8% (237 of 2851) of stool specimens from humans with gastrointestinal problems examined at the Hospital for Sick Children, Toronto, toroviruses were found by EM. Of these patients, 90% had diarrhea, 60% suffered from vomiting, 20% had abdominal pain, and 40% were anorectic; fever had preceded the gastrointestinal symptoms in 50% of the cases (M. Petric and M. Koopmans, unpublished observations, 1992). Epidemiological studies are under way to determine the significance of these findings.

#### IV. PATHOLOGY AND PATHOGENESIS

Postmortem inspection of the intestines of gnotobiotic calves after experimental infection with BRV exhibited few macroscopic changes, except for the thinness of the intestinal wall; on histological examination, changes typical of acute viral infection were noted. Villous fusion and atrophy (Fig. 6) and epithelial desquamation were seen, from the



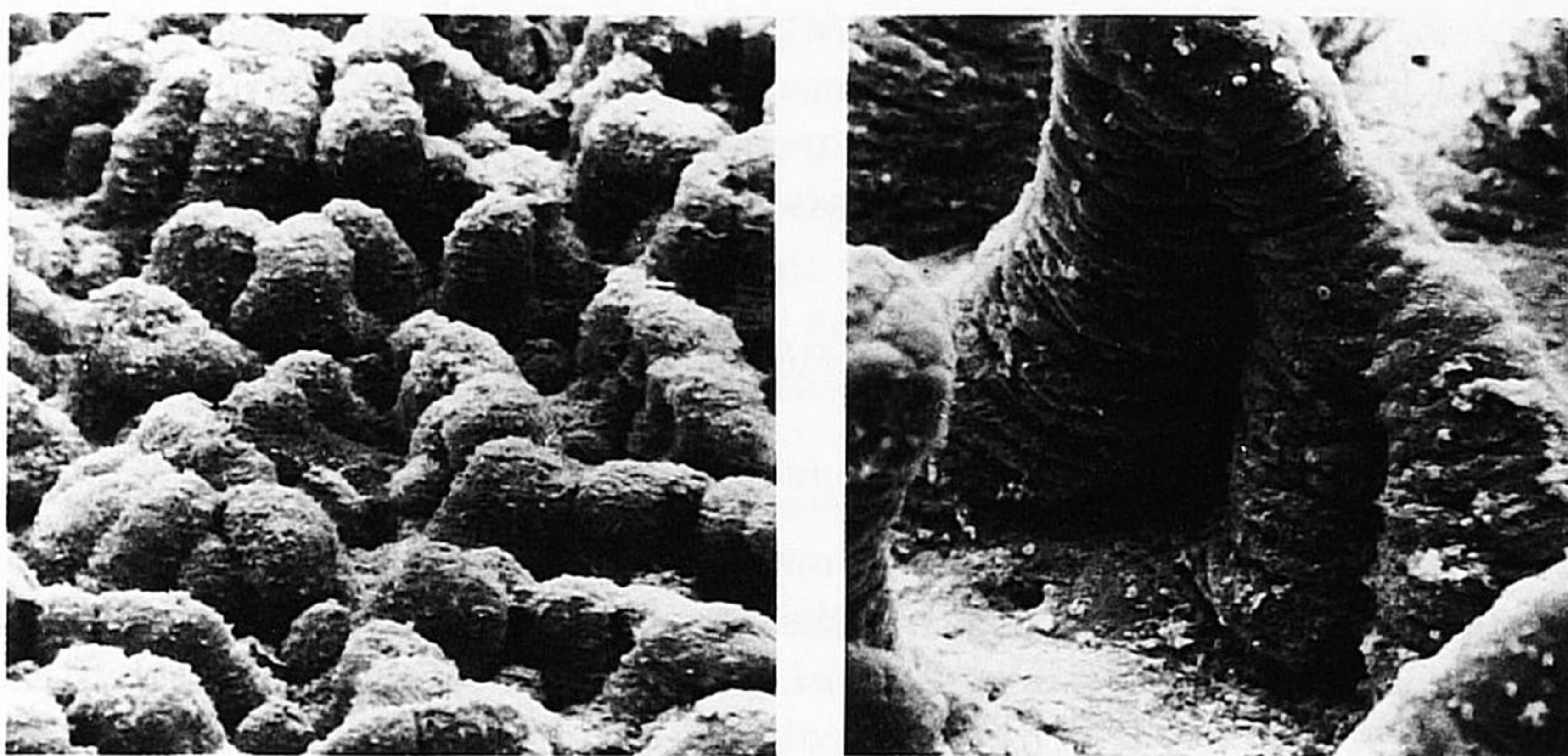


FIG. 6. Scanning electron micrographs of the small intestine of a BRV-infected gnotobiotic calf, showing severe stunting (left) and fusion (right) of normally finger-like villi.

midjejunum to the lower small intestine, in addition to areas of necrosis in the large intestine. Both crypt and villus epithelial cells were infected (Woode *et al.*, 1982; Woode, 1987). The watery diarrhea is probably a result of loss of resorptive capacity of the colonic mucosa, combined with mild malabsorption in the small intestine (Hall, 1987). Infection of crypt epithelium may affect the duration of diarrhea, as regeneration of villus epithelium starts in the crypts. The germinal centers of the Peyer's patches were depleted of lymphocytes and occasionally showed fresh hemorrhage (Woode *et al.*, 1982). The dome epithelial cells, including the M cells, had the same cytopathic changes that occurred in the absorptive cells of villi (Woode *et al.*, 1984; Pohlenz *et al.*, 1984). In tissues underlying the intestinal epithelium (lamina propria), the major pathological changes were edema and the presence of activated macrophages (Fagerland *et al.*, 1986). One of the BRV-infected calves had multifocal petechial hemorrhages and vesicular emphysema in the lungs (Woode *et al.*, 1982). EM examination of affected areas revealed the presence of virions in cells of both the small and large intestines. Extracellular virus appeared in close association with microvilli of absorptive cells and in coated pits between microvilli, which was interpreted to indicate receptor-mediated endocytosis (Fagerland *et al.*, 1986; Goldstein *et al.*, 1979). In addition, virions were found between enterocytes at the basal and lateral plasma membranes. Virions in various stages of degradation were present in macrophages within the lamina propria (Fagerland *et al.*, 1986; Pohlenz *et al.*, 1984).



In BRTV-infected calves laryngitis, tracheitis, and foci of pneumonia were noticed (Vanopdenbosch *et al.*, 1991, 1992b). No pathological data are available for the porcine, feline, bovine respiratory, and human infections with toroviruses.

## V. IMMUNITY

### A. Antigenic Relationships among Toroviruses

The infectivity of BEV is not neutralized by antisera against other known equine viruses, and this observation presented the first clue for a novel animal virus (Weiss *et al.*, 1983). Cross-reactions with BEV were observed with sera from calves that had been experimentally infected with BRV, as tested in neutralization tests (NTs) and enzyme-linked immunosorbent assays (ELISAs) (Woode *et al.*, 1982).

Apart from the original Breda isolate, two additional bovine strains have been reported; one was detected in feces from a 5-month-old diarrheal calf in Ohio, and a second Iowa strain was recovered from a 2-day-old experimental animal (Saif *et al.*, 1981; Woode *et al.*, 1985). On the basis of their reactivity in ELISA, immune electron microscopy (IEM), and hemagglutination/hemagglutination inhibition (HAHI) assays using rat erythrocytes, the three isolates were assigned to two antigenic types: BRV1, represented by the first Breda/Iowa isolate, and BRV2, comprising the Ohio isolate and the second Iowa isolate (Woode *et al.*, 1983, 1985). No antigenic cross-reactions with any of the known bovine viruses, including rota-, corona-, parainfluenza-, parvo-, and pestiviruses, have been found by IEM, HAHI, and IFA (Woode *et al.*, 1982, 1985).

Antigenic cross-reactivity between the BRV types and BEV exists at the P protein level; mouse immune serum raised against BRV2 virus recognized the polypeptides of the homologous virus and the two highest molecular weight proteins (105,000 and 85,000) of BRV1 virus in RIPA (Fig. 5, lane 2). The same serum inhibited HA of the heterologous serotype to a low but significant degree and efficiently neutralized the infectivity of BEV (Koopmans *et al.*, 1986). Since these experiments were done with surface-labeled virus preparations, no information was obtained about possible cross-reactivity at the level of the N protein. Two sera from cattle with antibody against Lyon-4 virus, a TVLP observed in feces from cattle in France (Moussa *et al.*, 1983), precipitated BEV proteins (including the N protein) from lysates of infected EMS cells in a RIPA (Horzinek *et al.*, 1985). In view of the notorious tendency of *Staphylococcus aureus* protein A to adsorb to the N protein (Fig. 4, left, lane 1) (Kaeffer *et al.*, 1989), these results must be reevaluated.



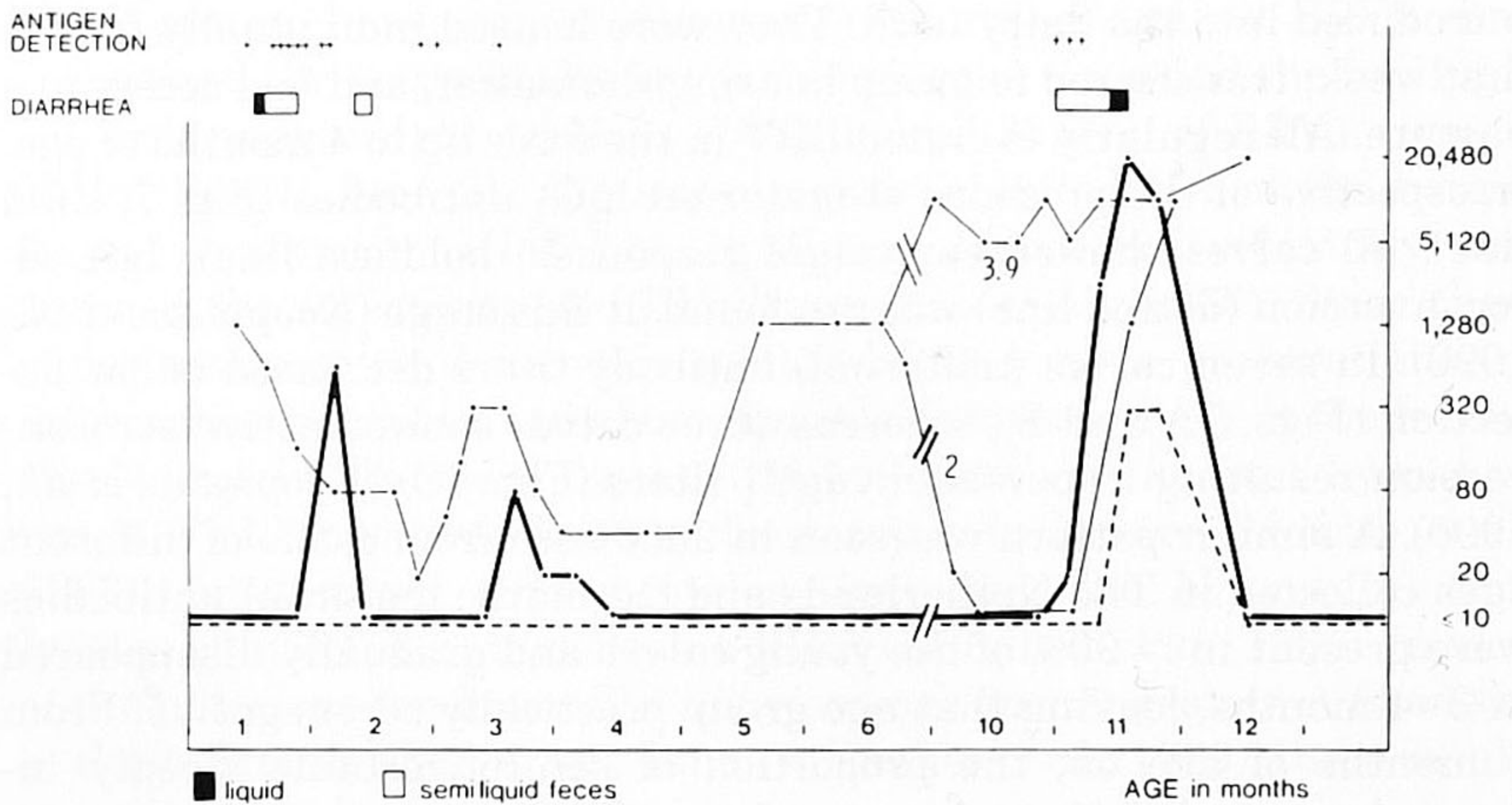
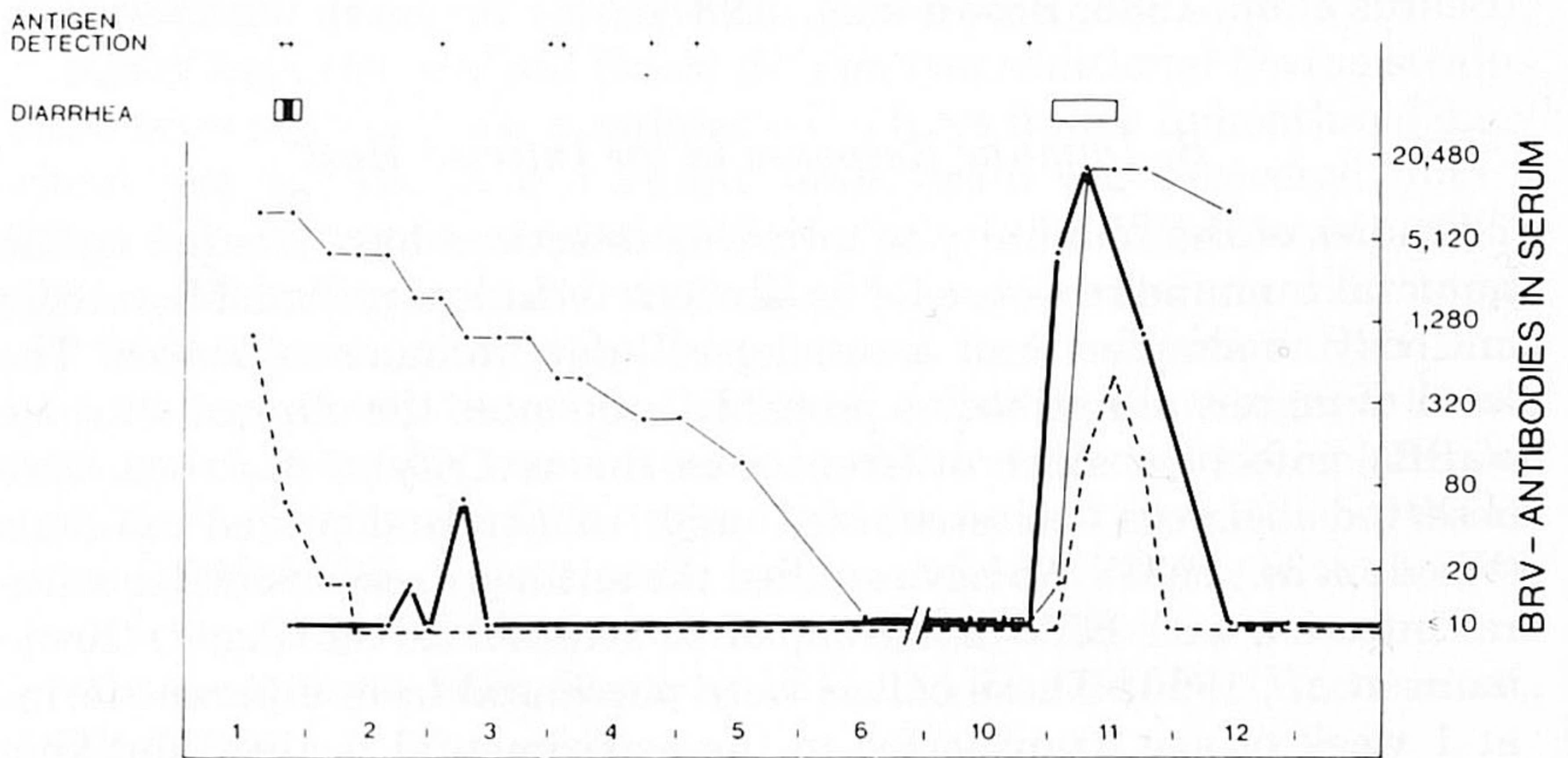
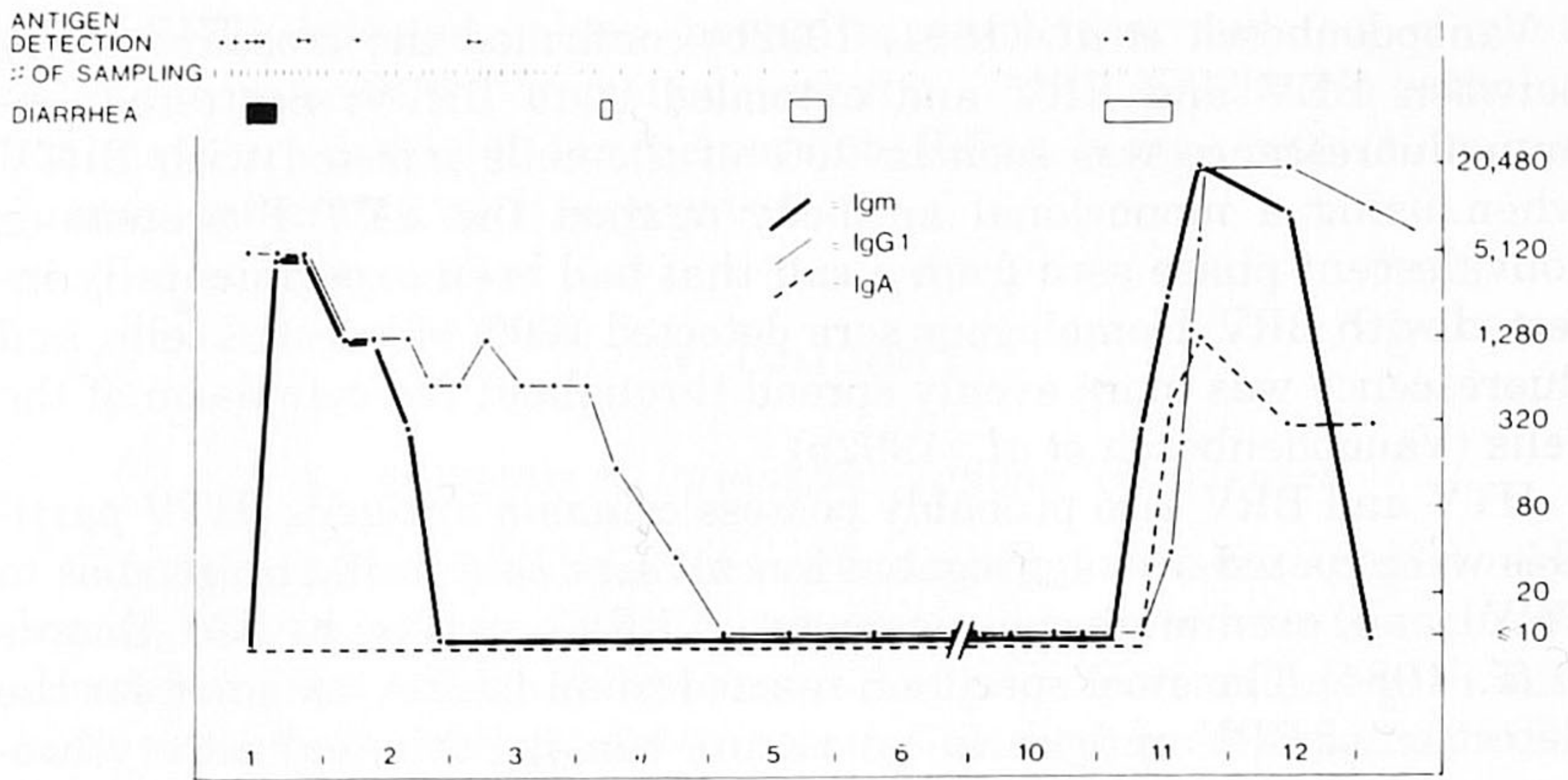
Vanopdenbosch *et al.* (1991, 1992b) confirmed the cross-reactivity between BEV and BRV and extended it to BRTV: Scattered immunofluorescence was seen in 20% of the cells infected with BRTV when using a monoclonal antibody against the BEV P protein or convalescent-phase sera from a calf that had been experimentally infected with BRV. Homologous sera detected 100% of infected cells, and fluorescence was more evenly spread throughout the cytoplasm of the cells (Vanopdenbosch *et al.*, 1992b).

HTV and BRV also probably possess common antigens. HTV particles were coated and aggregated by calf sera containing antibodies to BRV1 and, even more conspicuously, to BRV2, as seen by EM (Beards *et al.*, 1984). The stool specimen reacted in an ELISA designed for the detection of BRV antigen in calves and hemagglutinated rat erythrocytes at low titers; the HA was blocked by antisera to BRV1 and BRV2 (Beards *et al.*, 1986; Brown *et al.*, 1987).

### *B. Immune Response in the Infected Host*

Studies of the immunity to torovirus infections have focused on the humoral immune response for an obvious technical reason: Measuring antibody is simpler than assessing cellular immune responses. The level of maternal antibodies probably influences the clinical outcome of BRV infection, since differences in the severity of diarrhea were observed between colostrum-fed and colostrum-deprived animals (Woode *et al.*, 1985). We have studied the antibody response after natural infection with BRV in a group of 10 sentinel calves (Fig. 7) (Koopmans *et al.*, 1990). These calves were purchased from different farms at 1 week of age, transported to the experimental facility, and kept separated from other cattle until 10 months of age, when they were introduced into the dairy herd. They were housed individually for the first week, transferred to group housing thereafter, and had access to a pasture. All regularly excreted BRV in the feces up to 4 months of age. Irrespective of the presence of maternal IgG<sub>1</sub> antibodies (Fig. 7, solid line), all calves showed early IgM responses (boldface line); IgA seroconversion (dotted line) was not found at this stage (Koopmans *et al.*, 1990). In seven calves (maternal) antibody titers decreased below detection (Figs. 7A and B), whereas three calves showed active seroconversion resulting in persistent IgG<sub>1</sub> titers (Fig. 7C) (Koopmans *et al.*, 1990). A similar pattern was seen in 2000 sera from cattle of different ages collected in The Netherlands and Germany; maternal antibodies were present in > 90% of the young calves and gradually disappeared at 3–4 months, leaving that age group practically seronegative. From 7 months of age on, the proportion of seroconvertants steeply increased, to reach 94% at 2 years of age (Koopmans *et al.*, 1989).







After introduction of the sentinel calves into the dairy herd at 10 months of age, all of them had mild diarrhea, and shedding of BRV was observed in eight of them. Seroconversion for all antibody isotypes was observed, indicating lack of mucosal memory. In striking contrast, coronavirus infection in the presence of maternal antibodies led to an isotype switch in all calves but one, and a memory response was noticed after introduction into the dairy herd (Koopmans *et al.*, 1990). Differences in duration and severity between torovirus and coronavirus infections that might influence the degree of immune responses (Saif and Smith, 1985) were not detected. However, BRV has been reported to infect dome M cells, the epithelium overlying Peyer's patches (Pohlenz *et al.*, 1984; Woode *et al.*, 1984). Because M cells play an important role in local immunity of the gut, their degeneration—as seen after infection with BRV—might lead to an impaired immune response.

Vanopdenbosch *et al.* (1992b) studied the presence of antibodies to BRTV in veal calves from 7 days to 5.5 months of age; these authors did not find the decrease in antibody levels during the period that we observed in dairy calves (Koopmans *et al.*, 1989, 1990). This difference might be explained from different stabling conditions leading to potentially higher infectious pressure: Veal calves generally are kept in larger groups than dairy calves and do not have access to pasture.

The immune response in other animal species infected with toroviruses has not been studied.

## VI. EPIDEMIOLOGY

### A. Seroepidemiology

Torovirus infections are common. In cattle 90–95% of random serum samples contain antibodies when tested in BEV NT or BRV ELISA. Antibody-positive cattle sera were identified in every country examined: Belgium (Vanopdenbosch *et al.*, 1992a,b), Great Britain (Brown *et al.*, 1987), France (Lamouliatte *et al.*, 1987), Germany (van den Boom, 1986; Liebler *et al.*, 1992), India (Brown *et al.*, 1988), The Netherlands (van den Boom, 1986; Koopmans *et al.*, 1989), Switzerland (Weiss *et al.*, 1984), and the United States (Woode *et al.*, 1985).

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FIG. 7. BRV shedding, diarrheal episodes, and ELISA titers of BRV-specific antibody isotypes in sera of calves 1–5 (top), calves 6 and 7 (center), and calves 8–10 (bottom). Times of fecal sample collection are indicated by short vertical bars above graph A; sera were obtained at weekly intervals. (From Koopmans *et al.*, 1990.)



Most adult horses (81%) in Switzerland possess neutralizing antibodies to BEV (Weiss *et al.*, 1983, 1984). Seroconversions have been found in 9% of paired serum samples from individual adult horses that had been randomly collected, with intervals between bleedings ranging from 3 to 45 days ( $n = 273$ ; Weiss *et al.*, 1984). Furthermore, neutralizing antibodies to BEV were found in sera from goats, sheep, pigs, rabbits, and feral mice (Table II). Several investigators have looked for torovirus antibodies in sera from individuals that handle animals on a regular basis. No antibody to BRV or BEV was detected by ELISA or NT, respectively, in sera collected from veterinarians and farm workers in Great Britain ( $n = 92$ ) and Switzerland ( $n = 84$ ). Further, no antibodies were detected in 158 human and 38 simian sera collected in four villages in India with a high incidence of tropical sprue, a malabsorption syndrome of unknown etiology (Brown *et al.*, 1987, 1988; Weiss *et al.*, 1984). Zoonotic torovirus infections are therefore unlikely or infrequent. Also, the lack of detectable antibodies in humans and carnivores (Table II) may indicate that some TVLPs of humans and carnivores are more distantly related to BEV than are the ungulate toroviruses.

TABLE II  
PERCENTAGES OF ANIMALS CLASSIFIED ACCORDING TO SERUM NEUTRALIZATION TITER TO BERNE VIRUS<sup>a</sup>

| Animal species | No. of samples | Titer range |       |        |         |     |
|----------------|----------------|-------------|-------|--------|---------|-----|
|                |                | <10         | 10-50 | 50-100 | 100-200 | >20 |
| Ungulates      |                |             |       |        |         |     |
| Horses         | 507            | 19          | 29    | 16     | 15      | 21  |
| Cattle         | 129            | 14          | 29    | 25     | 18      | 14  |
| Goat           | 124            | 31          | 31    | 15     | 13      | 10  |
| Sheep          | 101            | 66          | 23    | 6      | 3       | 2   |
| Pig            | 112            | 19          | 52    | 12     | 10      | 7   |
| Carnivores     |                |             |       |        |         |     |
| Dog            | 46             | 100         |       |        |         |     |
| Fox            | 46             | 100         |       |        |         |     |
| Cat            | 107            | 98          | 2     |        |         |     |
| Lagomorphs     |                |             |       |        |         |     |
| Rabbit         | 80             | 79          | 21    |        |         |     |
| Rodents        |                |             |       |        |         |     |
| Mouse          | 26             | 20          | 65    | 15     |         |     |
| Primates       |                |             |       |        |         |     |
| Humans         | 84             | 100         |       |        |         |     |

<sup>a</sup>From Weiss *et al.* (1984).



### B. Disease Association

Our epidemiological surveys have shown the incidence of torovirus infections in two disease entities of cattle: diarrhea of breeding calves up to 2 months of age and winter dysentery of adult cattle in The Netherlands (Koopmans *et al.*, 1991c). Torovirus shedding was detected on 60% of the farms (15 farms tested) in the study, and in 4% of diarrheal calves ( $n = 187$ ), which was significantly higher than shedding detected in healthy calves (1%,  $n = 115$ ; Koopmans *et al.*, 1991c). The actual number of calves shedding BRV may have been higher, since ELISA-detectable virus shedding is limited to a few days (Koopmans *et al.*, 1991b; Woode, 1987).

The role of toroviruses in winter dysentery is controversial. Our study in The Netherlands found that seroconversion occurred significantly more often after winter dysentery outbreaks (149 cows on 19 farms) than on farms without a disease history (67 cows on eight farms). Looking at individual outbreaks, we found a significant number of torovirus seroconversions (>40%) in diseased cattle on three farms; coronavirus seroconversion was less common (Koopmans *et al.*, 1991c). Several investigators, however, have suggested a role for coronaviruses in winter dysentery. Van Kruiningen *et al.* (1985, 1987) detected coronaviral particles and antigen in lesions of the large intestines of cattle that had been infected with fecal specimens from cows with winter dysentery in the United States and found no seroconversions to toroviruses (van Kruiningen *et al.*, 1992). Several investigators have reported coronavirus shedding by adult diarrheic cattle (Benfield and Saif, 1990; Broes *et al.*, 1984; Espinasse *et al.*, 1981, 1982; Horner *et al.*, 1975; Saif *et al.*, 1988), but the same is true for healthy cattle (Collins *et al.*, 1987; Crouch and Acres, 1984; Crouch *et al.*, 1985). Seroepidemiological studies in Japan and the United States have added evidence that coronaviruses may indeed be involved: A high number of seroconversions was found in cattle with diarrhea (Akashi *et al.*, 1980; van Kruiningen *et al.*, 1992; Saif *et al.*, 1991; Takahashi *et al.*, 1980). Apart from the possibility that another, yet unidentified, agent causes winter dysentery, it is likely that infections with different enteropathogens can result in the same clinical disease picture, much like the situation with calfhood diarrhea.

Recently, another range of disease associations was published. Vanopdenbosch *et al.* (1992a) have confirmed the pathogenicity of BRTV for the respiratory tract and suggested a possible role in late abortion, central nervous system disturbances, sudden death, and in a syndrome resembling mucosal disease (in the absence of bovine viral diarrhea virus). The concept of localized toroviral infections restricted to the



enteric and/or respiratory system probably needs reconsideration. The same authors have found seven of 13 precolostral sera and 20 of 20 commercial batches of tissue culture-grade fetal calf serum to contain antibodies, indicating transplacental torovirus passage (Vanopdenbosch *et al.*, 1992b). In cases of abortion, toroviral antigen was detected by indirect IFA in cotyledons (Vanopdenbosch *et al.*, 1992a).

### C. Transmission

#### 1. Routes of Infection

BRV infection is probably spread through direct and indirect fecal-oral contact. In fecal preparations from experimentally BRV-infected calves, HA titers of  $3 \times 10^7$  units/ml have been measured (Woode *et al.*, 1983). The number of particles corresponding to 1.0 HA unit is unknown for BRV, but has been estimated at  $10^6$  TCID<sub>50</sub> for BEV (Zanoni *et al.*, 1986). When applying these figures to BRV, very high particle concentrations ( $10^{11}$ – $10^{12}$ ) may be expected in feces. Therefore, once an outbreak is under way, the infection can spread rapidly, especially if susceptible hosts are present (e.g., in the calving season).

In addition, the possibility of aerogenic infections can no longer be ignored, in light of recent reports about the isolation of BRTV from respiratory tract tissues (Vanopdenbosch *et al.*, 1991, 1992a,b). A dual tissue tropism has also been reported for bovine coronaviruses, which were detected simultaneously in the intestinal and respiratory epithelia from experimentally and naturally infected calves (Heckert *et al.*, 1991; Saif, 1987; Saif *et al.*, 1986). The duration of nasal shedding in these calves was almost twice as long as that of fecal shedding (Saif, 1987). Infection of nasal epithelial cells after oral and intranasal exposure of calves to BRV was reported (Saif and Heckert, 1990). Also, high numbers of seroconversions to BRV have been found in association with respiratory disease in calves at 3 months of age (Koopmans *et al.*, 1989). Virus-containing aerosols arising from all kinds of secretions and excretions, including feces, may be inhaled and eventually cause infections.

#### 2. Torovirus Susceptibility to Environmental Influences

Depending on the resistance of a virus to environmental influences, the surroundings of virus-shedding animals become more or less persistently contaminated. In general, enveloped viruses are quite unstable outside the host. However, BEV was found to be remarkably stable, even to the action of phospholipase C or deoxycholate and to pH values



ranging from 2.5 to 10 (Weiss and Horzinek, 1986a). This behavior resembles that of nonenveloped enteric viruses, and indicates that BEV may have adapted to passage through the gastrointestinal tract.

BRV1 appears to be less stable than BEV, because changes in its sedimentation behavior and density have been observed after prolonged storage at  $-70^{\circ}\text{C}$  (Koopmans *et al.*, 1986). The infectivity of a fecal preparation containing BRV1 was lost completely after 3 weeks at  $4^{\circ}\text{C}$  (G. N. Woode, Texas A&M University, Austin, TX, personal communication, 1986), whereas the BEV titer in cell-free supernatant from infected cells remained stable for 92 days under the same storage conditions (Weiss and Horzinek, 1986a). Two cycles of freeze-thawing of purified BRV2 resulted in loss of peplomers and in a large number of disintegrated virions, as tested by EM (M. Koopmans and W. Herbst, unpublished observations, 1988). The different storage conditions may explain the observed differences in the stability of BRV1 and BRV2; bovine toroviruses probably do not survive well outside their hosts, and other mechanisms for their persistence on farms are more likely.

### 3. Persistence of Infection in the Herd

The high prevalence of BRV antibodies in cattle herds (94%) cannot be explained by the few BRV infections found in calves (4%) and adult cows (14%) with diarrhea. The viruses may circulate through subclinically or chronically infected adult cows or calves. This is not unusual; repeated shedding of rota- and coronaviruses by adult cows has been detected by several investigators, using EM (Bulgin *et al.*, 1989; Collins *et al.*, 1987; Vanopdenbosch *et al.*, 1979), ELISA (Crouch and Acres, 1984; Crouch *et al.*, 1985), and IFA (Vanopdenbosch *et al.*, 1979). Both free virions and virus-antibody complexes have been detected, the latter more frequently (Crouch and Acres, 1984; Crouch *et al.*, 1985). A seasonal fluctuation has been described, with the highest numbers of coronavirus shedders at the time of parturition (Bulgin *et al.*, 1989; Collins *et al.*, 1987). Sixty percent of the calves of "carrier" cows developed diarrhea, as compared with 20% of calves from non-shedders. It is unknown whether toroviruses use similar mechanisms. Repeated torovirus shedding was observed in calves (Koopmans *et al.*, 1990), but not in antibody-positive adult cows. However, the use of more sensitive assays might show a different picture: Infections and seroconversions were observed in sentinel calves immediately after they had been stabled together with adult cows, testifying to their role as virus shedders (Koopmans *et al.*, 1990).

Reinfections with the same serotype, followed by antibody boosting, have been described in calves experimentally infected with ro-



tavirus (Schwers *et al.*, 1984), indicating a lack of protective mucosal immunity. Locally produced IgA—and in ruminants, IgG<sub>1</sub>—is important for preventing viral infections of intestinal villus and crypt epithelium [type I enteric infections (Saif and Heckert, 1990)]; circulating antibody therefore does not reflect mucosal immunity (Bienenstock *et al.*, 1981; Zaane *et al.*, 1986). Thus, animals possessing serum antibodies against BRV may be virtually nonimmune to (re)infection.

Alternatively, reinfection of an animal primed with a different serotype can lead to a booster response. Virions that have undergone antigenic changes at the level of the surface proteins may escape neutralization by IgA and IgG<sub>1</sub> in the gut lumen, thereby skirting the first line of defense against infection of the mucosal epithelium (Bachmann and Hess, 1983; Skehel and Wiley, 1986). Evidence for the existence of such a mechanism has been obtained by Goto *et al.* (1986), who repeatedly isolated rotavirus from the same calf at 1- to 2-month intervals and found that the isolates differed in neutralizability. Hamada *et al.* (1990) did the same for enteroviruses in adult cows and found a different T1 RNase fingerprint pattern for each isolate.

Antigenically different toroviruses can be expected. Two serotypes of BRV have been described (Woode *et al.*, 1985), and more probably exist. Viruses with an RNA genome can diverge rapidly because of the high mutation frequency which has, in part, been attributed to the lack of proofreading exonuclease activity in RNA replicases, resulting in the integration of mismatched bases into the nascent strand (Holland *et al.*, 1982; Steinhauer and Holland, 1987; Strauss and Strauss, 1988). The rate of mutation has been estimated at  $10^{-3}$  to  $10^{-4}$  per nucleotide per replication cycle. The actual rate of divergence (i.e., fixation in the genome of mutated residues) is limited by selection, since most mutations are deleterious. In a study of influenza virus isolates, the rate of divergence was 1% per year in the third codon (where many of the changes are silent), and 0.5% per year in both the first and second codon positions. Studies of other viruses have found divergence rates of 0.03–2% per year (Strauss and Strauss, 1988). Consequently, the RNA genome in a virus pool within an infected host cannot be described as a defined uniform structure, but rather as a weighted average of a large number of different individual sequences (the quasi-species concept) with one predominant genotype (Holland *et al.*, 1982; Steinhauer and Holland, 1987). Exposure to the host's immune system can result in a selective advantage for less abundant virus mutants. The unique BEV isolate is probably a torovirus host cell range mutant with the "selective advantage" to infect cultured cells from the horse and the mule.



## VII. DIAGNOSIS

A. *Virus Detection*1. *EM and Solid-Phase IEM*

EM and HAHl were initially used for torovirus detection in feces of cattle (Woode *et al.*, 1982, 1985), but they cannot be used in large-scale screening programs. Notoriously expensive and time-consuming, EM requires highly trained staff and, most importantly, careful interpretation of the images. Toroviruses are pleomorphic—intrinsically, but also depending on the particle orientation relative to the electron beam—and they may resemble coronaviruses (Woode, 1987). The subjective element in EM interpretation can be reduced by the introduction of a serological confirmation step, as in IEM (Woode *et al.*, 1985) or solid-phase immune electron microscopy (SPIEM) (Koopmans *et al.*, 1990; Liebler *et al.*, 1992). A “capture antibody” adsorbed to the grid in SPIEM may increase the sensitivity of detection of rotaviruses and enteroviruses approximately 30- and 60-fold, respectively, as compared with direct EM (Svensson *et al.*, 1983; Pegg-Feige and Doane, 1984), and 10-fold as compared with ELISA (Svensson *et al.*, 1983). For the detection of BRV, SPIEM (Fig. 8) and ELISA are almost equally effective, and few discrepancies were found when using the two tests [4% SPIEM-positive ELISA-negative samples and 1% SPIEM-negative ELISA-positive samples (Koopmans *et al.*, 1990; Liebler *et al.*, 1992)].

2. *Isolation in Cell Culture*

The isolation in equine cell culture of BEV from feces of a horse could never be repeated, although the infection must be quite common, given the high seroprevalence. The interpretation that the only *in vitro* isolate of BEV is a mutant is plausible, since reisolation from the same clinical material was successful, whereas several hundred attempts with material from other horses failed. Toroviruses replicate in the digestive tract. The epithelial lining of the intestine consists mainly of enterocytes, which are produced in the crypts and gradually differentiate while migrating up the villus. In BRV-infected intestinal epithelium, antigen-containing cells are detected by IFA in the zone between the upper third of the crypts and the villus top (Fagerland *et al.*, 1986; Pohlenz *et al.*, 1984). Thus, only cells which have attained a certain stage of differentiation appear to be infectable.

A stable human colon carcinoma line (CaCo-2) has been used as a model for torovirus infection of the gut. These cells resemble the epithelial lining of the intestine; after reaching confluency, they gradually differentiate, and at approximately 1 week after seeding they



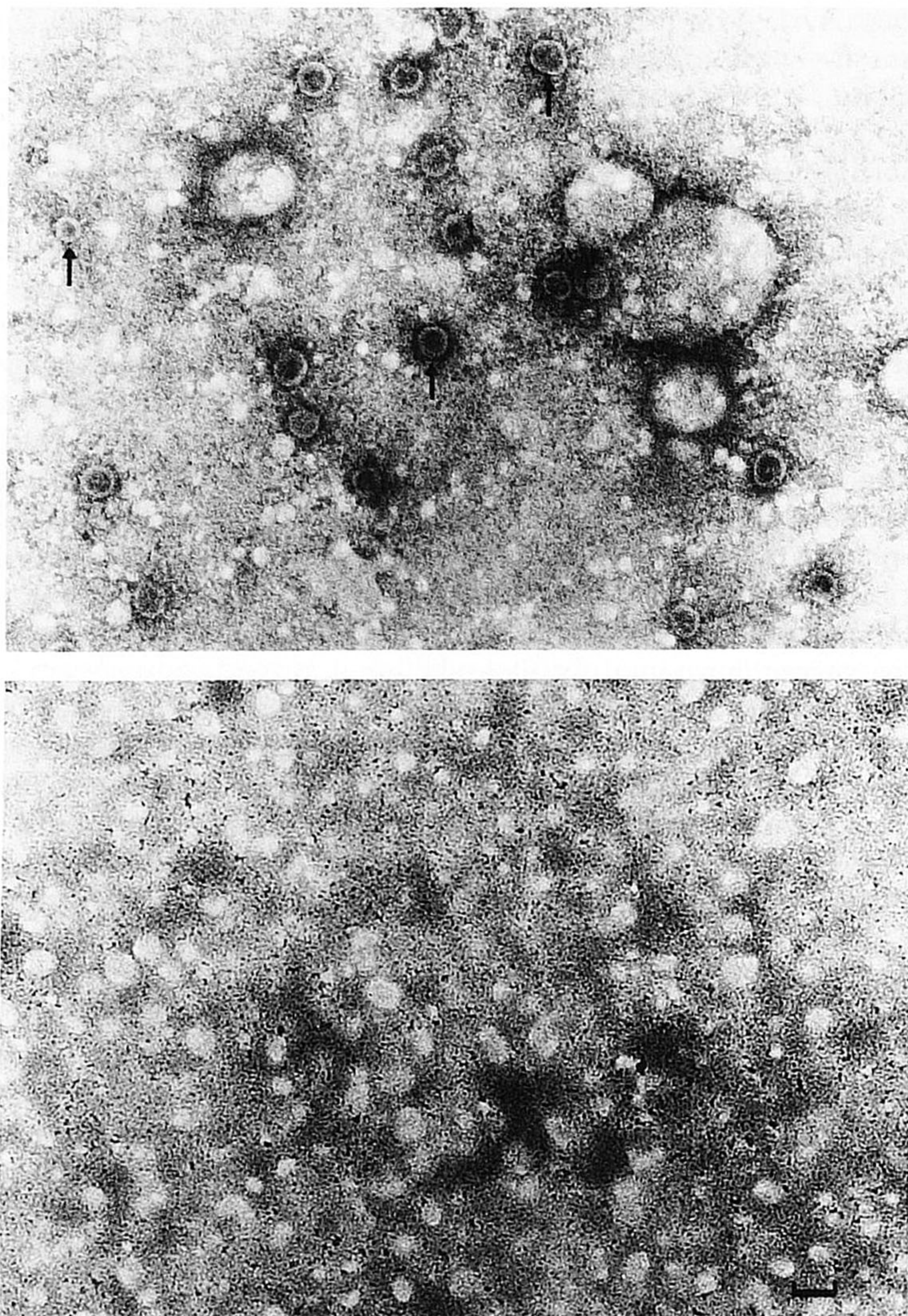


FIG. 8. Solid-phase immune electron microscopy (SPIEM) of feces from a gnotobiotic calf infected with BRV (arrows). Grids were precoated with calf hyperimmune serum against BRV (top) or cryptosporidia (bottom). Bar, 100 nm. (From Koopmans *et al.*, 1990.)

have developed into mature enterocytelike cells with a brush border and its associated enzymes (Pinto *et al.*, 1983). Indeed, low levels of BEV replication ( $10^4$  per ml) were obtained in CaCo-2 cells, but only in 3- to 9-day-old cultures. However, since cultivation of CaCo-2 cells is tedious and BRV replication could not be detected by immunohistol-



ogy, this approach has been discontinued (M. Koopmans and M. C. Horzinek, unpublished observations, 1988). Recent advances in the production and maintenance of intestinal epithelial cell lines would warrant a renewed effort in using them for torovirus cultivation (Peterson and Mooseker, 1992; Woodcock *et al.*, 1991).

The recently reported isolation of BRTV in cells from different animal species is very interesting but needs to be confirmed (Vanopdenbosch *et al.*, 1992b). Attempts at isolating HTV or FTV in cell culture were unsuccessful (Koopmans *et al.*, 1993b; Muir *et al.*, 1990).

### 3. HA Test and HI Confirmation

Both BRV1 and BRV2 possess a hemagglutinin for rat and mouse red blood cells (Woode *et al.*, 1982; Koopmans *et al.*, 1986), and BEV agglutinates human blood group O erythrocytes (Zanoni *et al.*, 1986). The specificity of the HA is confirmed by repeating the assay in the presence of immune and nonimmune sera (HAHI). However, the strong serotype specificity (Woode *et al.*, 1983, 1985) makes the HAHl test a poor candidate for screening purposes, as it ignores viruses with a different HA antigen. Also, normal fecal samples regularly exhibit nonspecific HA, which may be present at dilutions as high as  $2^6$  (Woode *et al.*, 1982); as a result, low BRV-specific HA titers are missed.

HA and HAHl have been used to identify BRV in sucrose gradient fractions (Beards *et al.*, 1986; Koopmans *et al.*, 1986). When the same techniques were used for HTV, a hemagglutinin for human blood group O erythrocytes was found in 50% of the EM-positive stools (Koopmans *et al.*, 1993b). Muir *et al.* (1990) reported the presence of a rat hemagglutinin in feces from cats with FTV.

### 4. ELISA

Since all attempts to grow BRV routinely in culture have failed, BEV grown in EMS cells has been tested for use as a heterotypic antigen in ELISA. However, virus titers were low and extensive background reactions with cellular material were seen despite purification using different methods (van den Boom, 1986). Therefore, BRV passaged in gnotobiotic calves and shed to high titer with their feces has been used (Woode *et al.*, 1982, 1985). The disadvantages of such a method are obvious; standardization is difficult, and false-positive reactions may arise [e.g., from "sticky" proteins such as bovine serum albumin that copurify with virions (van den Boom, 1986)]. Interference can also be expected from staphylococcal protein A, which binds to immunoglobulins of many species (Goudswaard *et al.*, 1978) and may react with the capture and detecting antibody used in ELISA (Chantler and Clayton, 1988). Finally, maternal or locally produced antibodies [a common situation in calves (Koopmans *et al.*, 1986,



1990)] may interfere with the binding of viruses to the immobilized and detecting antibodies (Chantler and Clayton, 1988). However, the speed of the ELISA and its adequacy for assaying large numbers of samples are important advantages, and it can indeed be performed with the aid of a feces-derived antigen, as long as proper controls are included.

Evidence is accumulating that HTV can be detected in ELISA using antibodies to BRV (Beards *et al.*, 1986; Koopmans *et al.*, 1993b). In a recently completed blinded study, 70% of the specimens that contained TVLPs by EM react positive in an ELISA using rabbit antisera to BRV2 as the detector antibody. Sera from rabbits immunized with purified HTV have recently become available (Koopmans *et al.*, 1993b) and need to be evaluated for use in immunoassays.

### 5. IFA Assay

Antigen of BRV can be detected by IFA as early as 28 hours postinfection in epithelial cells of the lower half of the villus and of the crypts of the affected areas (Woode *et al.*, 1982), as well as in dome epithelium (Woode *et al.*, 1984; Pohlenz *et al.*, 1984). Fluorescence is cytoplasmic (although a few nuclei may be faintly stained) and generally is highest in the intestines with the least tissue damage. The midjejunum is the first site to be infected, with viral infection progressing down the small intestine, eventually reaching the large intestine (Fagerland *et al.*, 1986). Therefore, diagnosis by IFA should be performed preferentially on sections of the large intestine, when post-mortem examination is done in calves that died after the onset of diarrhea (which is several days after the infection of epithelium).

Vanopdenbosch *et al.* (1991, 1992b) used hyperimmune sera from BRV-infected calves in IFA to detect viral antigen in lungs from BRTV-infected calves and for confirmation of cell culture isolation. In addition, these authors had prepared a serum in guinea pigs hyperimmunized with gradient-purified BRTV from cell culture.

### 6. Hybridization Assay

The RNA-DNA hybridization assay is not influenced by the host's immune status, has a high sensitivity, and does not require purified virus. cDNA probes covering portions of the structural protein genes and the polymerase gene of BEV all hybridize with BRV RNA under high-stringency conditions (>70% nucleotide sequence identity), indicating a high degree of sequence conservation. The 3' ends of BRV2 and BEV RNA, including a 70-nucleotide stretch of the BEV N protein gene, are 93% identical (Koopmans *et al.*, 1991b). Extrapolation to the



rest of the genome cannot be done, however, because replication is initiated at the 3' end, which therefore may be highly conserved (Hershey and Taylor, 1987). Our data confirm the close relationship between BEV and BRV, as suggested by their antigenic relatedness. To offer a comparison, the N protein genes of bovine and murine coronaviruses (MHV), which belong to the same antigenic cluster within the genus, are only 72% identical (Lapps *et al.*, 1987); identity between their spike protein genes ranges from 61% (5' end) to 74% (3' end) (Boireau *et al.*, 1990). The structural protein genes of two strains of MHV (A59 and JHM) are between 86.5 and 97% identical (listed by Luytjes *et al.*, 1987). When comparing the MHV figures with those for the short stretch compared in toroviruses, BRV and BEV must be considered strains of the same virus.

The hybridization assay has been used to detect BRV RNA in clinical fecal samples from infected calves and was shown to correlate well with ELISA test results (Koopmans *et al.*, 1991b). A disadvantage for routine application is the requirement of isotopes. However, methods for enzymatic labeling of cDNA have been reported, and biotinylated or digoxigenin-labeled probes (Kumar *et al.*, 1988; Nago *et al.*, 1988) may replace radioactive preparations, with comparable sensitivity and specificity.

### 7. Reverse Transcriptase Polymerase Chain Reaction Amplification

The reverse transcriptase polymerase chain reaction amplification (RT-PCR) provides a further increase in the sensitivity of torovirus detection. It has been established to amplify genomic RNA from BEV and BRV from purified virus preparations, fecal specimens, and tissues. When the conserved 3'-end genomic sequences of BEV and BRV are used, the sensitivity of detection by RT-PCR alone is  $10^2$  TCID<sub>50</sub>/ml for purified BEV and  $10^3$  TCID<sub>50</sub> for BEV contained in feces. Subsequent hybridization with an internal oligonucleotide probe increased the level of sensitivity at least 10-fold (Koopmans *et al.*, 1993a). Given the unneeded high sensitivity of the test, the risk of contamination, the high levels of virus that are usually shed, and the availability of ELISA reagents, the RT-PCR is not our first choice for routine virus detection. However, for the tracing of virus carriers, pathogenesis studies, elucidation of tissue tropism, and identification of the sites of virus latency and of virus shedding in body fluids other than stool specimens, the technique may prove very useful. With regard to viral RNA detection in tissues, *in situ* RT-PCR should be explored as a powerful method, combining high sensitivity with tissue localization.



### *B. Antibody Detection*

#### *1. NT*

Tissue culture-adapted BEV has been used in an NT for the screening of sera from a wide range of species (Weiss *et al.*, 1984; Brown *et al.*, 1988). Seroconversion has been demonstrated when paired horse sera were tested. It is unknown whether the NT can be used to diagnose recent infection in species other than horses.

#### *2. HI Test*

The BRV HA induces antibodies that can be detected in an HI test (Woode *et al.*, 1985). After injection of BRV1 or BRV2 in mice, cross-reacting antibodies were detected using the HI test, but only at low serum dilutions (Koopmans *et al.*, 1986). This serotype specificity (Woode *et al.*, 1985) makes the HI test a poor candidate for screening purposes, since it overlooks infection with viruses that possess a different hemagglutinin.

#### *3. ELISA*

A blocking ELISA has been used to detect BRV-specific antibodies of all isotypes (Beards *et al.*, 1986; Koopmans *et al.*, 1989). However, the presence of maternal antibodies in the sera from most young calves results in positive reactions, which make the test unsuitable for diagnostic use in this age group. A BRV-specific IgM detection ELISA cannot be recommended for routine diagnostic purposes in young calves because of the variability in the occurrence and maximum values of the IgM peaks, as observed in sentinel calves (Koopmans *et al.*, 1990). Thus, virus detection methods appear to be the only answer in the pursuit of the diagnosis of torovirus infection in young calves.

## VIII. TAXONOMY

Similarities between toro- and coronaviruses have sparked a spirited discussion about their taxonomic position that eventually resulted in the classification of both as genera within the family Coronaviridae. The information that led to this decision is reviewed here.

Superficially, a morphological resemblance exists between toro- and coronaviruses, which led to the initial description of toroviruses as "coronavirus-like." Coronaviruses (for a review see Spaan *et al.*, 1990) are enveloped positive-stranded RNA viruses. In negatively stained preparations virions are pleomorphic, roughly spherical, measuring 60–220 nm in diameter. They have characteristic club-shaped surface "peplomer" projections 12–24 nm long, which are arranged as a halo of



radiating spikes (corona) around the particle. Bovine coronaviruses often display a second fringe of shorter peplomers (Pensaert and Callebaut, 1978). The typical tubular nucleocapsid, which sometimes can be seen in negatively stained torovirus preparations [especially when grids are reexamined after a few days' storage (Beards *et al.*, 1986)], is not seen in coronaviruses.

The difference in the size of their N proteins was the first physical difference emphasized between toro- and coronaviruses (Horzinek *et al.*, 1984). Coronaviral N proteins have a characteristic  $M_r$  of 43,000–50,000 (Masters and Sturman, 1990); toroviruses, 18,300 (Snijder *et al.*, 1989). In addition, the absence of any significant sequence similarity between structural protein genes of toro- and coronaviruses was taken as evidence to underline their separate taxonomic positions.

On closer analysis after the nucleotide sequences of most toroviral genes had become available, fundamental similarities between toro- and coronaviruses were revealed. The triple membrane-spanning toroviral E protein is considered structurally equivalent to the coronaviral M protein, again without any amino acid sequence similarity (Snijder *et al.*, 1990b; den Boon *et al.*, 1991a). The P protein of BEV resembles the coronaviral peplomer (S) protein not only in terms of morphological characteristics. Both are N-glycosylated, are of about the same size, and contain a trypsinlike cleavage site (although not present in all coronaviruses), heptad repeats, and hydrophobic domains in comparable positions. In addition, the shape of the surface projections and the formation of dimers indicate that the spikes may have similar tertiary and quaternary structures. Within the coronavirus genus the amino acid sequence of especially the C-terminal half of the spike protein is highly conserved (de Groot *et al.*, 1987). Since there are no such sequence similarities between BEV P and coronaviral S proteins, convergent evolution could be invoked to explain the comparable organization and structure of the BEV P protein. The absence of antigenic relationships and amino acid sequence homologies is indicative of a large evolutionary distance between the two genera. However, the discovery of a number of conserved domains in the polymerase genes of corona- and toroviruses indicates that they are evolutionarily related (Snijder *et al.*, 1990c).

The overall amino acid sequence identity between ORFs 1b of BEV and coronavirus polymerases is approximately 20%, as compared with 56% identity between two coronaviruses. However, four domains are rather well conserved between toro- and coronaviruses: two regions common to RNA polymerases (domain 1, the GDD motif; domain 3, the helicase motif; 45–50% identical amino acids), a metal-binding "finger" structure (domain 2), and a region that seems to be unique for



toro- and coronaviruses (domain 4; 40–45% identical amino acids) (Bredenbeek *et al.*, 1990; Snijder *et al.*, 1990c, 1991).

Also, with respect to the genome organization and expression, resemblances with coronaviruses became evident (Snijder *et al.*, 1990a,c, 1991). The toroviral genes encoding the polymerase protein and the structural proteins are present in the same order (5'—polymerase—peplomer—membrane—nucleocapsid—3'). They are expressed from multiple subgenomic mRNAs which form a 3'-coterminally nested set (Snijder *et al.*, 1990a,c). A difference is the apparent lack of a common leader sequence at the 5' end of the torovirus mRNAs, which coronaviruses do possess (Snijder *et al.*, 1990a). The polymerase protein gene of both viruses consists of two overlapping ORFs (1a and 1b); the latter is expressed after translational frame-shifting that results in a fusion product of the two ORFs. Thus, it had to be concluded that toro- and coronaviruses are ancestrally related, and toroviruses are now accommodated as a second genus within the family Coronaviridae (Table I) (Pringle, 1992).

This taxonomic assignment appears neither illogical nor does it violate traditional classification principles, by which structural criteria have been considered important and practical for a long time. However, there is a recent tendency to also include arteriviruses in the Coronaviridae family. This cluster of positive-stranded RNA viruses includes lactate dehydrogenase-elevating virus, simian hemorrhagic fever virus, the Lelystad/porcine reproductive and respiratory syndrome virus (Plagemann and Moennig, 1992; Wensvoort *et al.*, 1991), and equine arteritis virus, its eponymic representative. The latter is the *de facto* prototype and has been extensively characterized by the Utrecht laboratory. The virion is spherical, about 70 nm in diameter, and possesses an isometric, probably icosahedral, nucleocapsid surrounded by a tightly adherent lipoprotein membrane, thereby fulfilling the structural criteria of togaviruses (Horzinek *et al.*, 1971). On the other hand, it owns a nested set of subgenomic RNAs, a frame-shifting mechanism in translation of the ORF 1a/1b region, and a gene suite like those of the Coronaviridae family members (den Boon *et al.*, 1991b). Assigning the arteriviruses to this family on the basis of some similarities in replication would be no less fallacious than continuation of the togavirus assignment. Classification of arteriviruses as a new family would appear to be an adequate solution.

## IX. FUTURE RESEARCH

Even though rapid progress has been made in torovirus research during the last decade, much remains to be done. A few urgent ques-



tions should be mentioned: (1) The role of toroviruses in disease in humans needs to be addressed in epidemiological studies, especially since preliminary evidence has shown that they are quite prevalent. (2) Attempts should be made to adapt HTV to human cells such as CaCo-2 or HRT, because successful isolation would open the way for further characterization of these viruses. (3) Similarly, the importance of BRTV should be studied, not only to confirm their role as a potential pathogen in cattle (Vanopdenbosch *et al.*, 1991, 1992a,b), but also for their ability to replicate in cell culture, since this feature enables the molecular characterization of a second torovirus. (4) The sequence analysis of BEV should be completed to obtain a genomic map of the prototype torovirus. (5) A diagnostic application that has not yet been tested is the use of BEV probes for *in situ* hybridization, which would have similar applications as the IFA test, but also could be used to detect toroviruses in tissues from other species (including humans, Koopmans *et al.*, 1991a), provided that highly conserved sequences are used as probes. (6) At the Utrecht laboratory expression products of individual BEV genes are being developed and tested for use in a new generation of diagnostic assays, which would be very helpful in the previously mentioned epidemiological studies.

#### ACKNOWLEDGMENTS

The authors thank Dr. M. Petric (Hospital for Sick Children, Toronto, Ontario, Canada) and Dr. C. Humphreys (Centers for Disease Control and Prevention, Atlanta), Dr. M. Weiss (University of Berne, Berne, Switzerland), and Dr. K. Holmes (Uniformed Services University of Health Sciences, Bethesda, Maryland) for the electron micrographs of HTV, BEV, and human coronavirus, respectively; we thank Dr. G. Woode (Texas A&M University, Austin, Texas) for the photographs of intestines from infected calves. We also gratefully acknowledge the editorial assistance of J. O'Connor and the help of many colleagues who made information available to us prior to publication, or who critically reviewed the manuscript.

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