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Toroviridae: a taxonomic proposal*

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With 6 figures and one table

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Introduction

In 1972, a virus was isolated during routine laboratory diagnostic work from a horse under observation at the surgery clinic in Berne, Switzerland. The animal died about one week after the sample had been collected showing pseudomembranous enteritis and miliary granulomas and necrosis in the liver upon post mortem examination; *Salmonella lille* was considered as the causative agent. Berne virus (BEV; laboratory designation of the strain: P 138/72) was not neutralized by diagnostic antisera against notorious equine viruses and was shown to possess a unique morphology and substructure (10). Neutralizing antibodies to BEV were found in the Swiss cattle and horse population (9).

In 1982, Woode et al. (14) reported studies with an unclassified virus isolated from diarrheic calves in Breda, Iowa, USA. Breda virus (BRV) was propagated in gnotobiotic calves and caused agglutination of rat erythrocytes; indications for the existence of two serotypes of BRV were obtained from hemagglutination inhibition experiments. — An antigenically related virus was isolated from cattle in Lyon, France, in calf testicle cultures (LYV; 4); in immunofluorescence tests it cross-reacted with BEV antibody positive horse sera and LYV antibody positive cattle sera neutralized BEV (10). Very recently (1) particles resembling BE/BR viruses in morphology were described in the stools of children and adults with gastroenteritis in Birmingham, England (BIV) and Bordeaux, France; by immune electron microscopy, the structures were obviously related with BRV. Particles of a similar morphology were seen in stool specimens of children with gastroenteritis in Rotterdam, The Netherlands, in 1979 and probably earlier (G. J. P. Schaap, unpublished results, Fig. 1).

Apart from this morphologic evidence, indications have been obtained using serum neutralization tests that antibodies to BEV are prevalent in ungulates, lagomorphs and two species of wild rodents (11).

These observations indicate that BE/BR-like viruses occur in different species including man and that they may be pathogenic. The present review is the first attempt to

* This article is affectionately dedicated to Prof. Dr. Z. Dinter on the occasion of his 70. birthday.

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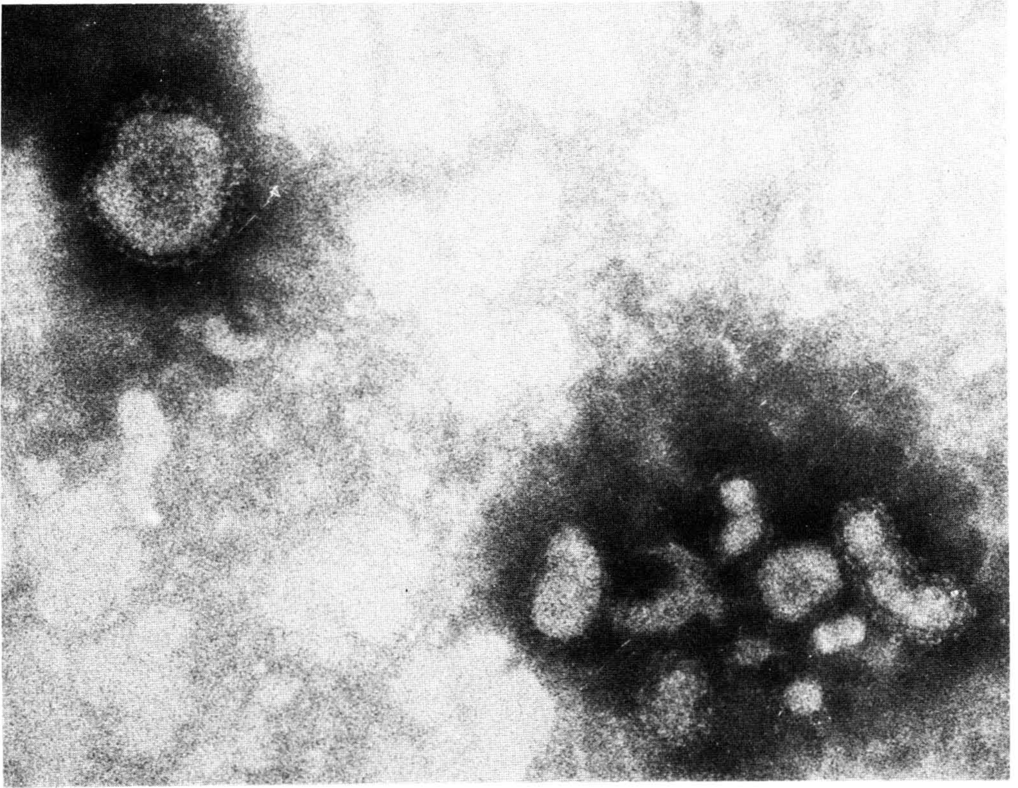

Fig. 1. Particles of torovirus morphology encountered in the feces of a 3-year-old child with gastroenteritis. Enlargement about 170,000 fold, phosphotungstate stain. Courtesy of Dr. G. J. P. SCHAAP, GGD Rotterdam, The Netherlands

summarize and discuss the available (partly unpublished) data and to report the unique physico-chemical and biological characteristics of BEV, the only isolate studied in culture so far. On the basis of the available information the establishment of a new family of animal viruses has been proposed at the 6th International Congress of Virology at Sendai, Japan. Pending the decision of the International Committee on Taxonomy of Viruses the name *Toroviridae* has been coined for the new family (2) and will be used throughout this publication.

Cultivation

The P 138/72 strain of BEV was isolated in secondary horse kidney cells, inoculated with material from a rectal swab; the same material yielded the virus on two subsequent reisolations. Significantly, this has remained the only equine torovirus strain until now, although some hundred isolation attempts were made. Rapid adaptation of the P 138/72 strain to equine dermis (E. derm.) and embryonic mule skin (EMS) cells was observed where the virus caused a pronounced cytopathic effect (Fig. 2), provided that the monolayer was subconfluent at the time of infection. In view of the wide-spread occurrence of neutralizing antibodies against toroviruses in cattle (11), fetal calf sera to be used in tissue culture for virus isolation must be pretested.

We have studied some 20 established cell lines of different origin for their ability to support multiplication of BEV; all attempts were negative. Also BRV could not be adapted

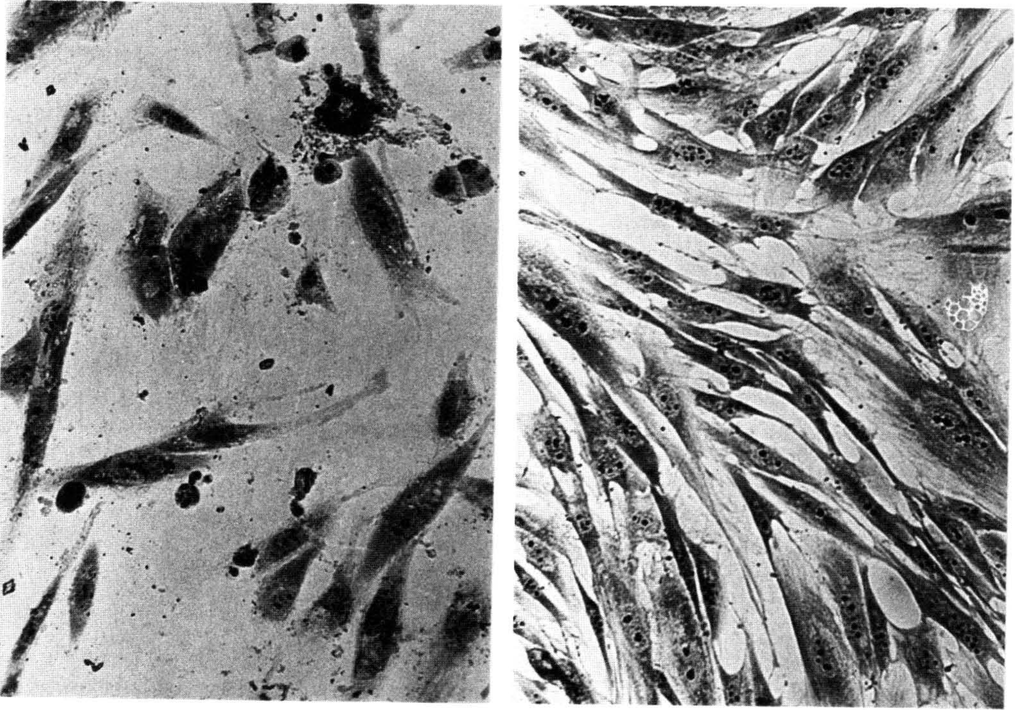

Fig. 2. Embryonic mule skin (EMS) cells 24 hours after infection with Berne virus at a multiplicity of >3 (left) and uninfected (right). Note detachment from the support and pyknosis. Giemsa stain

to growth in culture until now and must be propagated in calf-to-calf passages using gnotobiotic or colostrum deprived animals (14).

The cytopathic properties of LYV in calf testicle cell culture are doubtful in view of our observation of another virus-like particle (isometric, about 20 nm, without envelope) in material of this strain (Egberink and Horzinek, unpublished results).

Purification

Berne virus is easily purified and concentrated using a two step procedure. Supernatants from infected cultures are mixed with equal amounts of a saturated solution of ammonium sulphate and kept overnight at 4 °C. There is no need for pH control in view of the extraordinary stability of BEV (12) covering a range between 2.8 and 9.7; The precipitate contains the entire infectivity of the starting material but only about 10 % of the contaminating protein. The resuspended precipitate is clarified by low speed centrifugation and layered on top of a 15—50 % sucrose gradient; upon equilibrium centrifugation, a main infectivity peak is encountered at a density of about 1.16 g/ml. Recovery of infectivity, however, does not exceed 3 % in the gradient fraction and < 10 % if the whole gradient is considered. The decrease in infectivity is probably due to a loss of surface projections during centrifugation (10). The purification procedure has been successfully applied to suspensions of feces from BRV-infected calves (Horzinek and Woode, unpublished observations).

When preparations with a lower degree of purity are acceptable (e. g. for use as antigen in ELISA), precipitated and resuspended material can be layered on a 15/50 % sucrose cushion and spun into the interphase layer. Alternatively, pellets obtained after

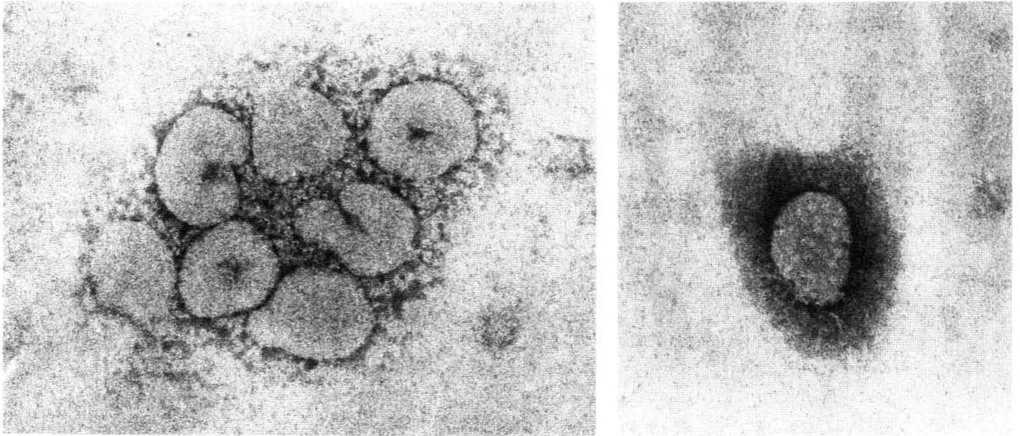

Fig. 3. Group of purified Berne virus particles showing the variations in morphology (left); a dotted appearance of the negatively stained virion surface is sometimes observed (right), indicative of end-on views of peplomers. Enlargement about 130,000 fold, phosphotungstate stain

ultracentrifugation (60,000 x g, 90 min.) of BRV-infected intestinal contents can be used for morphological analysis and as antigen in ELISA (WOODE, personal communication). Performance indices for centrifugation should be based on a sedimentation coefficient of about 450 S for the infectious virion (EDERVEEN and HORZINEK, unpublished results).

Morphology

Since most reports of toroviruses are based on observations of particles in stools or feces, the negatively stained virion has been extensively described (BRV: 14; LYV: 4; BIV: 1). Torovirions are pleomorphic particles of a spherical, oval, elongated or kidney-shaped morphology, measuring 120 to 140 nm in diameter. In preparations of purified virions we have observed a sausage-like internal structure with transverse striations (estimated periodicity about 4.5 nm) which appeared tightly attached to the membrane and did not leave the particle when the membrane was damaged. Depending upon the preparation virions are either bald or studded with projections (peplomers).

There is some controversy about the morphology and dimensions of the peplomers. We have described them as "drumsticks" consisting of a thin stalk carrying a distal spherule (total length about 20 nm; 10). WOODE et al. (14) have recorded surface projections of 7.6—9.5 nm in BRV. The particles of BIV were described as carrying peplomers 7—9 nm in length. Occasionally what appeared to be a second ring of smaller peplomers was seen, partly superimposed upon the first (1). The longer peplomers (17—24 nm) are believed by WOODE et al. (14) to be of doubtful specificity and have been reported only occasionally on particles in samples of human feces (1). — We have observed dots similar in size and distribution to end-on views of the "drumstick"-peplomers when the flat surface of a virion faced the electron beam and was defined by the negative stain (Fig. 3).

The thin section morphology of toroviruses was studied so far only with BEV (in infected equine cells: 10) and BRV (in gut epithelium from infected calves: 5). BEV-infected cells revealed densely staining spherical, elliptical and elongated particles accumulating at the cytoplasmic membrane and in vacuoles. At higher magnification, a clear distinction can be made between an electron-lucent envelope and a dark core. Rod- and crescent-shaped cores are prevalent in virions in the extracellular space or in cytoplasmic vacuoles. Twin circular structures with a conspicuous light centre which we interpret as cross-sections through a hollow, tubular nucleocapsid (diameter 23 nm) bent into an

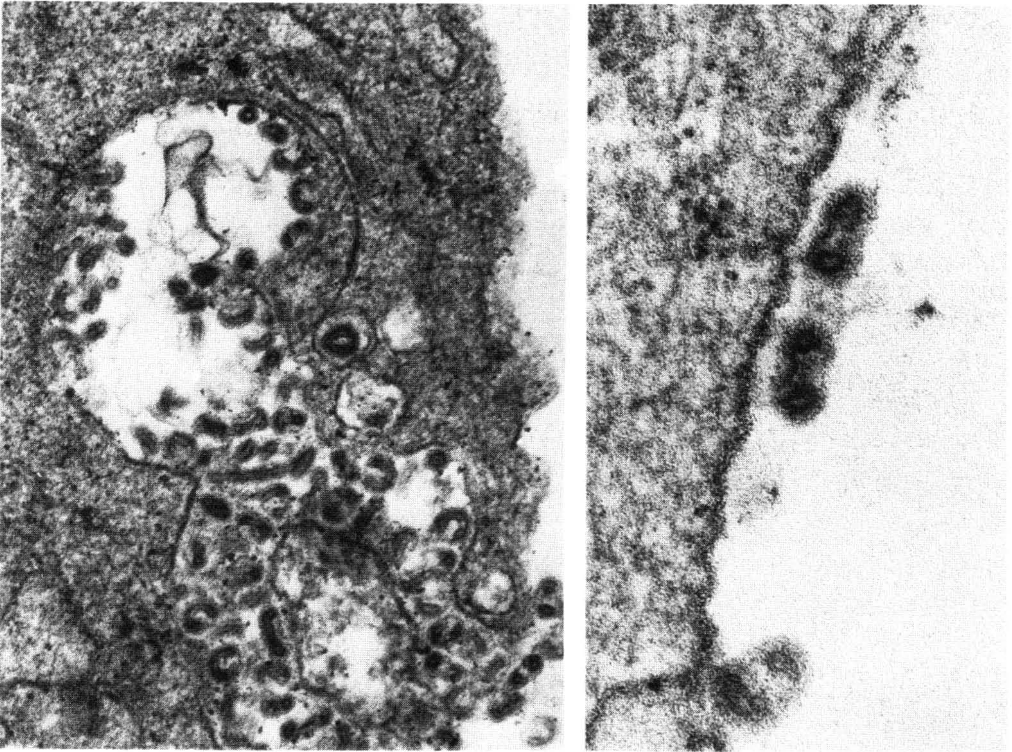

Fig. 4. Accumulations of Berne virus particles (enlarged about 70,000 fold) in a thin section through an infected EMS cell; torus-, C- and komma-shaped virions, rods and twin circular structures are visible (left). At a higher magnification (about 170,000 fold, right) virions adsorbed with their flat surface to the cytoplasmic membrane can be observed in cross section showing the hollow, tubular nucleocapsid bent into a torus. Note the defined distance of the virions from the membrane (about 20 nm) indicative of peplomers

open torus are regularly seen (Fig. 4); they are surrounded by a tightly fitting membrane. The length of the tubular structure can only be approximated; the calculated average value (104 ± 16 nm) is meaningless in this respect since it only reflects the frequency distribution of orientations in space of particles. A better estimate is our maximum value of 171 nm but the true length of the capsid may be slightly greater (180 nm, when assuming a non-overlapping 23 nm-tube filling the space within a discoidal envelope with an inner diameter of 80 nm). — From the asymmetric distribution of the diameters of all opaque internal structures of circular or elliptical shape in thin-section electron micrographs we concluded that the virus particle is not spherical in shape (10).

In cells of the intestinal mucosa of BRV-infected calves, elongated enveloped virions with rounded ends were detected, their average dimensions being 35 × 80 nm. The particles were described as being pleomorphic and varying in length (5). The crescent shape described above for BEV was not reported.

The morphological data are best explained by assuming a helical nucleocapsid, tightly coiled into a hollow tube which is either straight or bent into an open torus. Fig. 5 represents a synthesis of the images obtained from negatively-stained and thin-sectioned virus preparations. A tightly fitting envelope surrounds the core. It is our impression that its curved shape is maintained by the membrane; with this assumption the virion would

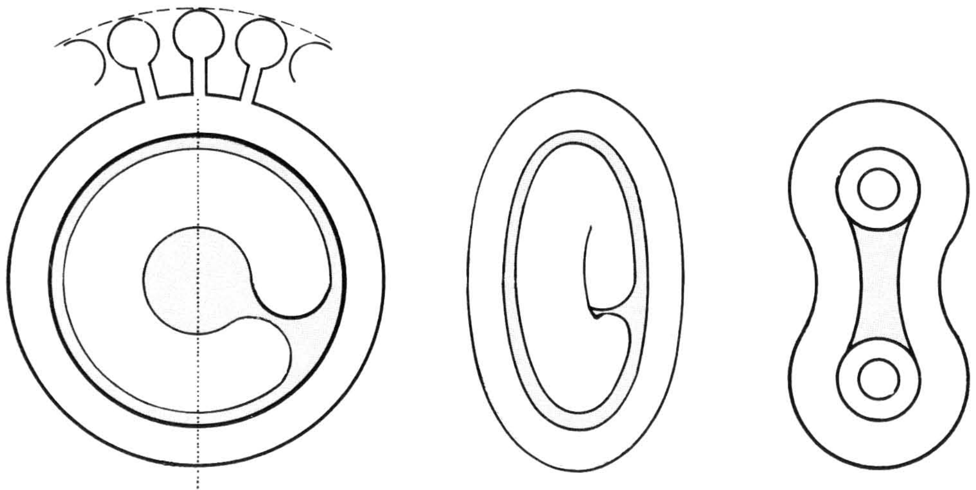

Fig. 5. Synthesis of torovirion architecture as compiled from electron micrographs of negatively stained and thin-sectioned particles

assume the morphology of a biconcave disk — not unlike an erythrocyte — as visualized in our cross-sections. Alternatively, the envelope could follow the smaller curvature of the torus, thereby creating a sausage- or kidney-shape. Further support for an elongated tubular nucleocapsid, probably of helical symmetry, comes from the observation of circular cross sections with an electron-lucent center, of enveloped bacilliform particles and of convoluted strand structures (in the nucleus of infected cells, see Fig. 6).

A protean particle morphology — bacilliform, kidney-shaped or discoidal — is quite uncommon in virology; different orientations of the particles with respect to the electron beam increase the heterogeneity of the picture still further. This is certainly the main reason why electron microscopists were reluctant to accept the pleomorphic structures as viral in nature, when encountering them e. g. in fecal specimens. — It cannot be excluded that the morphology of BEV is the consequence of the *in vitro* passage history of this isolate and that the elongated or kidney-shape (which is prevalent in micrographs of BRV) represents the "natural" structure; for orthomyxoviruses this phenomenon has been reported. Alternatively, toroviruses may be less restricted in their morphopoietic pathways, perhaps budding sideways as well as longitudinally. No convincing images of budding virions have been obtained so far.

Chemical composition

Toroviruses possess a lipoprotein membrane, as evidenced by electron microscopy, by their low buoyant density in sucrose (1.16 g/ml), and their sensitivity to organic solvents (10).

The presence of an RNA genome is indicated by the observation that growth of BEV was unaffected by the presence of IUDR under conditions where a DNA virus was inhibited. Preliminary evidence suggests that the genome is single-stranded; experiments to determine its size, colinearity and polarity are being performed.

We have analyzed the structural polypeptides of BEV. The major protein (as evidenced by ^{14}C amino acid labelling) is a 20 K species which is phosphorylated. Second in abundance is a 22 K protein. Additional species of 17 K, 37 K, and 80 to 120 K have been found in ^{35}S-methionin-labeled purified virus preparations and in immune precipitates employing extracts from infected cells. The 20 K species is the main nucleocapsid protein; together with a 17 K polypeptide, it occurs in a high density structure which can be liberated from BEV and extracted from infected cells using detergent treatment; the 22 K

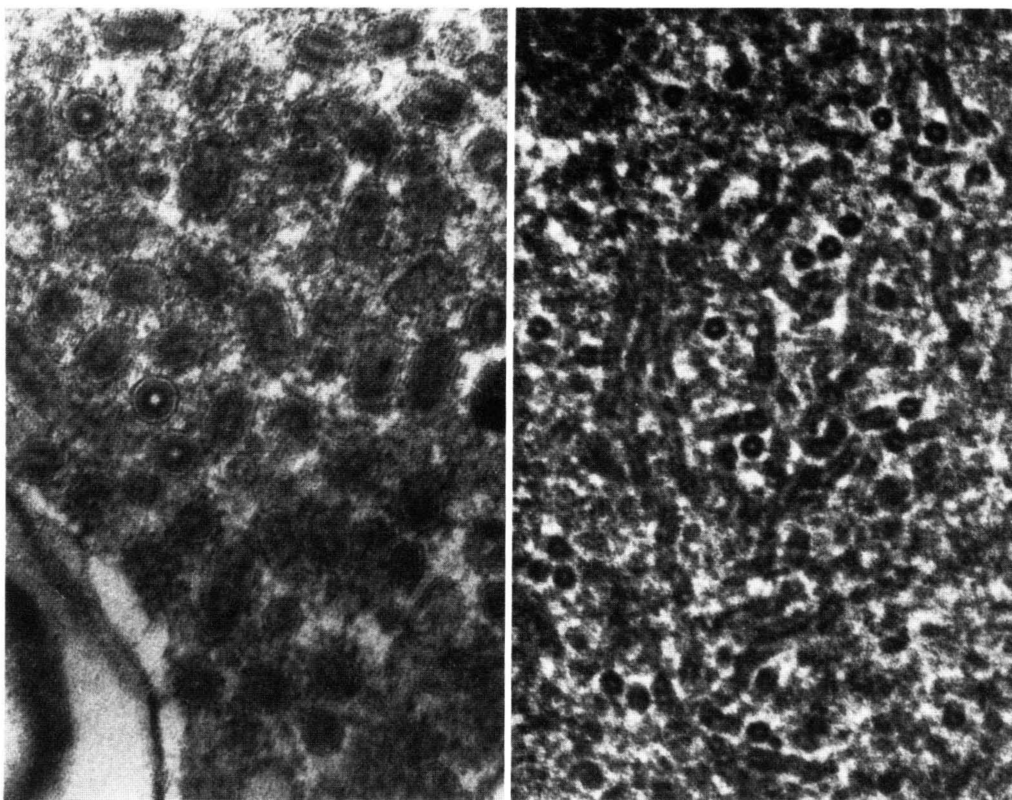

Fig. 6. Electron micrographs of thin sections through enteroabsorptive cells of the ileum from a calf after infection with Breda virus (enlargement about 160,000 fold). Longitudinal and transversal sections across enveloped, tubular ("hollow") structures in cytoplasmic vacuoles (left) and tubules in the nucleoplasm (right) are shown. The nuclear tubules lack an envelope and possess a smaller diameter. (Courtesy of Drs. J. F. L. POHLENZ and G. N. WOODE, Ames, Iowa, USA)

polypeptide is the main envelope protein. The 37 K species (which is phosphorylated to a significantly higher degree than the 20 K polypeptide) possibly constitutes a matrix protein. The heterogeneous label distribution in the 80—120 K range is indicative of glycosylation. It may be assumed that several polypeptides are found in this region and that they are constituents of the peplomer (3; HORZINEK and EDERVEEN, unpublished observations).

Antigenic structure

Since infectivity can be titrated only of BEV so far, the discovery by WOODE et al. (14) of a hemagglutinating activity in BRV preparations was an important finding. Rat erythrocytes are agglutinated over a wide pH range and the activity can be specifically inhibited by immune sera; elution and red cell lysis were reported. Using this technique the authors were able to distinguish between two isolates provisionally termed serotypes. Additional virus strains have been found in Ohio (7), one of them related to BRV serotype 2. The high specificity of the hemagglutination-inhibition test will have to be exploited for the antigenic definition of torovirus serotypes in the future. Attempts to show a hemagglutinating activity for BEV were without success; however, this may have quantitative rather than intrinsic reasons. — Experiments are under way to identify the polypeptide responsible for the hemagglutinating activity.

Also the ELISA procedure has been found useful in torovirus studies. For monitoring of viral activities in sucrose gradients, fractions can be bound to microtiter polystyrene wells after dilution in carbonate buffer. The indirect modification of the test using an anti-BEV horse serum and a rabbit anti-horse peroxidase conjugate was employed (10). The technique can be readily adapted for seroepidemiologic studies (11) — Using a similar procedure, WOODE et al. (13) have coated polystyrene microtiter plates with BRV from calf feces semipurified by ultracentrifugation; cross reactions between the serotypes were observed, but the titers in the homologous reactions were 6—10 fold higher as compared to the heterologous reactions (7).

Although not suitable for routine purposes, radioimmune precipitation using ^{35}S-methionin-labeled infected and uninfected cellular extracts is a powerful tool for monitoring antibody specificities in sera from different animals. The nucleocapsid protein of 20 K seems to be preferentially precipitated by heterologous antibody (HORZINEK and EDERVEEN, unpublished results).

Virus replication

Indirect immunofluorescence tests have been performed on gut sections of BRV-infected calves using homologous sera. The host cell spectrum appears to be very narrow. Intracytoplasmic fluorescence of epithelial cells has been reported only in sections of the jejunum, ileum and spiral colon, mainly in the lower half of the villi and extending into the crypts. No positive cells were observed in subepithelial tissue nor in the epithelium of the anterior small intestine (14).

In BEV-infected EMS cells, single cycle replication was completed in about 12 hrs;

Table 1

Percentages of animals positive in seroneutralization of Berne virus (from WEISS et al., 1984; reproduced by kind permission from Elsevier Science Publishers, Amsterdam, The Netherlands)

	numbers of samples	Titer range <10	10 - 50	50 - 100	100 - 200	>200
Ungulates						
horse	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	63	17			
Rodents						
mouse*	26	20	65	15		
Primates						
man*	84	100				

* In cats, one serum sample from the Netherlands and one from Switzerland reproducibly showed titers of 10 and 14, respectively. Low but reproducible titers were obtained with sera from laboratory rabbits. All (7) sera of the wild mouse species *Apodemus flavicollis* had titers of < 10 whereas the (2) *Clethrionomys* samples titered 46 and 94, respectively. Of the 17 samples from *A. sylvaticus*, only one had a value below 10. — Testing of the human sera at low dilutions resulted in neutralization values of < 10 in all 84 samples; 63 of these samples showed values < 2 upon re-examination. Erratic inhibition of virus growth was observed with 8 sera (calculated "titers" between 2 and 7).

yields and cytopathology are very much dependent upon the confluency of the monolayer, with low titers resulting in contact inhibited cultures. Viral proteins appeared about simultaneously from the 6th hour after infection onward. In pulse experiments using ^{35}S methionine viral proteins were detected in the nuclear extract in addition to the cytoplasm (HORZINEK et al., unpublished results). The observation that virus replication is inhibited by actinomycin D and alpha-amanitin is additional evidence that a synthetic step in cellular metabolism is needed for torovirus replication. Also UV-irradiation of the cells before infection with BEV lead to dramatic decreases in virus yields (3). Recently, our conception of a nuclear involvement in torovirus replication has received support by the observation of tubular 23 nm-structures in the nucleoplasm of intestinal cells from BRV-infected calves (5; see Fig. 6); in uninfected cells, these have never been observed (CHEVILLE, personal communication). Hence, nuclear membranes are likely to be involved in the morphopoiesis of toroviruses, comparable to the genus Lyssavirus and the plant subgroup B of rhabdoviruses. A predominantly perinuclear fluorescence has been observed by the Ames, Iowa group of workers (WOODE, personal communication). — A rabbit serum containing antibody mainly against the 20 K protein of BEV (as evidenced by radioimmune precipitation) resulted in preferential labelling of the nucleus of infected EMS cells (biotin-avidin technique; EDERVEEN and HORZINEK, unpublished observations).

Seroepidemiology

Using the neutralization test, sera from horses, cattle, goats, sheep, pigs, dogs, cats, red foxes, laboratory rabbits, three species of wild mice (*Apodemus sylvaticus, A. flavicollis* and *Clethrionomys glareolus*) and from man were studied. Table 1 shows that significant antibody titers and percentages were encountered in all ungulates, in two rodent and one lagomorph species. Inconclusive results were obtained with feline and human sera (11). The discriminative potency of virus neutralization (and of hemagglutination inhibition) is very high and a more group reactive assay is needed for showing the actual prevalence of antigenically related toroviruses in different species of animals.

Some experience using ELISA for seroepidemiology has been gained; QUESADA et al. (6) have found 88.5 % of randomly collected cattle sera (n = 156) in the USA positive for BRV antibodies; details of the technique have been described (13). Also 8 out of 14 horse sera tested gave positive results (WOODE, personal communication). We have tested more than a hundred horse, cattle and pig sera against BEV in ELISA and found the method suitable for routine serology (WEISS et al., unpublished observations).

Concluding remarks

The study of toroviruses is hampered by the difficulty of growing them in culture. In animals, however, they may reach extremely high concentrations. BRV has been reported to attain hemagglutinin titers of 2^{20}; under the assumption that about 10^5 physical particles are required for 1 hemagglutinating unit, virus concentrations exceeding 10^{11} can be expected. Electron microscopy therefore appears to be a suitable technique also for diagnostic purposes. Torovirus replication in the gut is favoured by an unusually great pH- and protease-stability of infectivity (as shown for BEV: 12) and an actively dividing, vast and rather homogeneous cell population in the intestinal tract. A breakthrough in cultivating these agents must be awaited before the BEV results can be confirmed in other virus-cell systems. — Isolation of BEV was most probably a singular chance event involving a spontaneous host range mutant.

When discussing our negatively stained electron micrographs of purified BEV with colleagues experienced in the identification of enteric viruses, it was repeatedly confirmed that similar particles are not uncommon in fecal material from humans and animals. Because of their ill-defined morphology, however, they were either regarded as non-viral in nature or compared with coronaviruses, to whom they bear a superficial resemblance; this has brought forth designations such as "corona-like" viruses, "minicorona" viruses

etc. which henceforth must be avoided. We were able to show that BEV has a unique pattern of structural polypeptides (3). For coronaviruses, the molecular weight of about 50 K of the nucleocapsid protein (8) is considered of taxonomic importance for a definition of the family; in contrast, BEV possesses a major capsid polypeptide of 20 K (HORZINEK and EDERVEEN, unpublished results).

Replication of coronaviruses is independent from host cellular functions as they can be grown in enucleated cells and in the presence of actinomycin D (8). This is in contrast to toroviruses where indications for a nuclear involvement during virus replication have been obtained using electron microscopy (in BRV: 5; see Fig. 6), inhibitors of transcription, UV irradiation of the host cell, and immunohistology (in BEV: 3; EDERVEEN and Horzinek, unpublished observations).

Although the definition of the viral genome, e. g. its size, strandedness (polarity) and structure (cap, poly[A]-tail) are still unknown, the results obtained so far justify a taxonomic initiative. A family status is suggested for the antigenically related viruses demonstrated in horses, cattle and man. The term "Toroviridae" is proposed (2), from "torus" meaning a "surface or solid shaped like a doughnut and formed by revolving a circle about a line in its plane without intersecting it" (WEBSTER's Third International Dictionary, 1976). The nucleocapsid of BEV as it is encountered in thin sections through infected cells, in suspensions of positively stained (uranyl acetate) particles and in ether- or detergent-treated purified virions may show such a morphology.

At the present state of knowledge (and extrapolating largely from the study of BEV), we should like to propose the following provisional definition of the Toroviridae family:

"Toroviruses are enveloped RNA viruses containing an elongated tubular nucleocapsid of presumably helical symmetry. The capsid may be bent into an open torus, conferring a disk- or kidney-shaped morphology to the virion (largest diameter 120—140 nm) or straight, resulting in a rod-shaped particle (dimensions 35×170 nm). The lipoprotein membrane is studded with projections; a hemagglutinating activity has been demonstrated. Major structural proteins of 22 K and 20 K have been identified, the latter occurring in the capsid. Replication is dependent upon some nuclear function of the host cell. Evidence of infection with toroviruses has been obtained in ungulates, lagomorphs, rodents and humans; indications for enteric infection and/or disease exist for equines, bovines and humans."

Note added in proof: The RNA of BEV is probably of positive polarity; a cDNA probe prepared against polyA-positive RNA from infected cells hybridized with genomic virion RNA (HORZINEK *and* EDERVEEN, *unpublished results).*

Summary

A new family of viruses is proposed for pleomorphic particles observed in feces of animals and men. This article summarizes the present knowledge about the properties of toroviridae. One strain — Berne virus, isolated from a horse — can be propagated in equine cell cultures.

Infections with these viruses seem to be widespread in animals.

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Zusammenfassung

Toroviridae — ein Vorschlag zur Taxonomie

Eine neue Virusfamilie wird für pleomorphe Partikel vorgeschlagen, die im Kot von Mensch und Tier nachgewiesen wurden.

Diese Übersicht faßt das derzeitige Wissen über die Eigenschaften der Toroviridae zusammen. Ein Virusstamm — Bern Virus, das von einem Pferd isoliert wurde — läßt sich in der Zellkultur vermehren.

Infektionen mit diesen Viren scheinen beim Tier häufig zu sein.

Résumé

Toroviridae: une proposition de taxonomie

Une nouvelle famille de virus est proposée pour des particules pléomorphes, mises en évidence dans des matières fécales humaines et animales. Cet aperçu comporte les connaissances actuelles sur les propriétés des Toroviridae. Une souche virale, Virus de Berne, isolée d'un cheval, peut se cultiver en culture cellulaire.

Des infections avec ces virus semblent fréquentes chez l'animal.

Resumen

Toroviridae: propuesta taxonómica

Se propone una nueva familia taxonómica de virus para partículas pleomorfas que han sido puestas en evidencia en las heces y estiércol de personas y animales.

Este artículo epitoma el conocimiento actual sobre las propiedades de los Toroviridae. Una estirpe virósica — el virus Berna, aislado de un caballo — se puede multiplicar en cultivo celular.

Las infecciones con estos virus parecen ser frecuentes en los animales.

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