Zb 12

Journal of Veterinary Medicine Series B
Zentralblatt für Veterinärmedizin Reihe B

Infectious Diseases, Immunology, Food Hygiene, Veterinary Public Health

Edited by / Herausgegeben von

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VOLUME/BAND 33, 1986

228 Figures/Abbildungen
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PAUL PAREY Scientific Publishers Berlin and Hamburg

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Verantwortlich gemäß Berliner Pressegesetz: Prof. Dr. DDr. h. c. A. Mayr, Veterinärstraße 13, D-8000 München 22

Verlag und Druck: © Verlagsbuchhandlung Paul Parey, Berlin und Hamburg 1986. Anschrift: Lindenstr. 44—47, D-1000 Berlin 61 (West), Tel.: 2 59 90 40, Telegr.-Adr.: Pareyverlag Berlin. Telex 18 47 77. Printed in Germany by Saladruck, Köpenicker Str. 18—20, D-1000 Berlin 36.

This journal is covered by Biosciences Information Service of Biological Abstracts, Current Contents (Series Agriculture, Biology & Environmental Sciences), Science Citation Index and Automatic Subject Citation Alert of Institute for Scientific Information, Index Medicus and Medlars of National Library of Medicine, Chemical Abstracts (selectively), Nutrition Abstracts and Reviews of the Commonwealth Bureau of Animal Nutrition, Index Veterinarius and The Veterinary Bulletin of the Commonwealth Bureau of Animal Health, and Vetdoc of Derwent Publications Ltd.

ISSN 0721–1856

J. Vet. Med. B 33, 155—159 (1986)
© 1986 Paul Parey Scientific Publishers, Berlin and Hamburg
ISSN 0721—1856

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Occurrence and Isolation in Tissue Culture of Equine Rotaviruses*

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With 2 figures and 2 tables

(Received for publication June 4, 1985)

Summary

During an outbreak of diarrheal disease in foals on a stud farm in north Germany rotavirus was demonstrated by 1 or more of 4 tests in 6 out of 24 samples tested. From one of the 6 positive samples equine rotavirus was isolated in cell culture. This isolate belonged to group A of rotaviruses and was classified in subgroup I.

Introduction

Rotaviruses are known to cause diarrhoeal disease in many mammalian species, including man. In all species examined to date, the infection is strictly localized to the enterocytes of the small intestine. Infected cells lose their microvilli and become vacuolated, resulting in shortening and atrophy of the villi. This general feature of rotaviral infection has also been observed in foals (Conner and Darlington, 1980).

Equine rotaviruses have been observed in many parts of the world, e. g. in the USA (Conner and Darlington, 1980), Australia (Studdert *et al.*, 1978), New Zealand (Durham *et al.*, 1979) and in Japan (Imagawa *et al.*, 1982). In Europe, their presence has been reported from Great Britain (Flewett *et al.*, 1975) and Ireland (Strickland *et al.*, 1982). Thoroughbred mares from France were shown to have rotavirus antibodies in their serum (Conner and Darlington, 1980). However, only a few strains of equine rotaviruses — all originating from the USA — have been successfully isolated and propagated in tissue culture (Hoshino *et al.*, 1983 a, b; Gillespie *et al.*, 1984). In this report we describe the occurrence and isolation of equine rotavirus from Germany.

Material and Methods

Case History

On a stud farm in the north of Germany diarrhoea of suckling foals has been observed since 1981. On this farm, 25 foals were usually born per year and kept on grass in three groups. Diarrhoea

* Dedicated to Prof. Dr. P. A. Bachmann († May 25, 1985).

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Table 1. Results of rotaviral diagnosis of 6 out of 24 samples, which were positive by at least one test

	ELISA	IEM	RNA-PAGE	Isolation in cell culture
576/84	+	+	+	+
567/84	+	+	+	−
589/84	+	+	+	−
651/84	−	−	+	−
1203/84	−	−	+	−
293/85	+	Nt	−	−

Nt = Note tested

was observed in June or July, and lasted 10—18 days, except in 1981, in which year the foals and their dams were kept in the stable during the night (as it was extraordinarily cold during this season). In 1981, diarrhoea occurred in May and lasted three weeks.

Diarrhoea was always characterized by grey faeces resembling watery concrete, loss of appetite and exsiccosis.

The percentage of affected foals increased from 48 % in 1981 to 80 % in 1984. In 1981 and 1982 all animals survived the disease, whereas in 1983 and 1984 one and two foals, respectively, died from diarrhoea. In 1984 and 1985 24 samples were assayed for rotavirus.

Demonstration of rotavirus-antigens by ELISA

Faecal samples were diluted 1 : 5 in PBS, sonicated for 10 seconds and clarified by centrifugation. The supernatants were used for ELISA, IEM, RNA-PAGE and virus isolation in cell cultures.

ELISA was performed as a double sandwich assay essentially as described by BACHMANN (1979). The rotavirus subgroup was determined using subgroup specific monoclonal antibodies in an indirect ELISA (HOSHINO *et al.*, 1983 a)[3].

Immune electron microscopy (IEM)

Clarified supernatants were mixed with group-specific hyperimmune serum prepared against bovine rotaviruses and sedimented onto carbon-coated copper grids. The grids were stained with 2 % phosphotungstate and one grid per sample was examined in a Zeiss EM 10 electron microscope.

Demonstration of rotaviral RNA by PAGE

Supernatants (300 µl) were extracted with an equal volume of water-saturated phenol; RNA in the aqueous phase was precipitated with three volumes of ethanol at − 20 °C. After centrifugation for 5 min at 12,000 rpm the pellet was dissolved in Laemmli's sample buffer (LAEMMLI, 1970) and applied on 7.5 % polyacrylamide gels with 2.5 % stacking gels. The gels were prepared as described by LAEMMLI (1970), but BIS was replaced by DATD for cross-linking. RNA segments were separated overnight at 50 V and stained with silver as described by MERRIL *et al.* (1981).

Isolation of equine rotavirus in cell culture

Supernatants positive for either ELISA, IEM or RNA-PAGE were first treated with acetylated trypsin (Sigma, Munich; 20 µg/ml) for 30 min at 37 °C. The activated material was then absorbed on Ma 104 cells in tubes for 60 min. After washing, the cells were fed with EMEM containing 2 µg/ml acetylated trypsin. The tubes were incubated in a roller apparatus and inspected daily for CPE. After 7 days, viral replication was monitored by ELISA.

For serial propagation of cell culture-adapted rotavirus, the initial trypsin activation was omitted. Cell culture adapted equine rotavirus was concentrated by precipitation with PEG 6000 and purified by fluocarbon extraction. Virus was then pelleted and the resuspended pellet was layered on top of a preformed CsCl gradient (density 1.3—1.42 g/ml). The gradient was spun for 2 hours at 100,000 × g and visible bands were collected by side-puncture.

[3] Monoclonal antibodies were a gift from Dr. H. B. GREENBERG, NIH, Laboratory of Infectious Diseases, Bethesda, Md, USA.

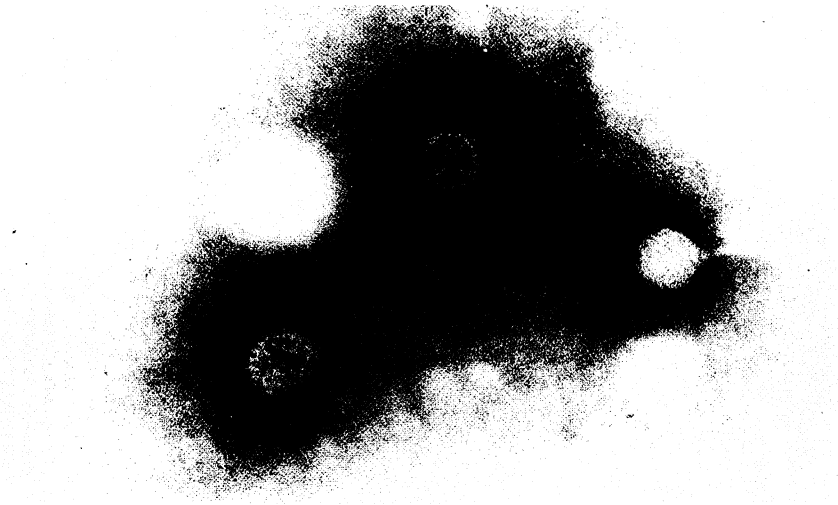

Fig. 1. Rotavirus particles from the original faecal extract no. 576/84. Note that the particles are not aggregated by group specific immune serum, probably because antigenic sites were blocked by faecal antibodies. (120,000 ×)

Results

Demonstration of rotavirus in the faeces of diarrheic foals

In 6 out of 24 samples one or more of the tests used for detection of rotavirus was positive. In three cases the results of ELISA, IEM and RNA-PAGE were all identical (Table 1). In three cases only one of the tests was positive. Sample 1203/84 was detected only by IEM, no. 293/85 was scored positive only by ELISA. When testing no. 651/84 typical rotaviral RNA-patterns were seen in PAGE, but IEM and ELISA were negative.

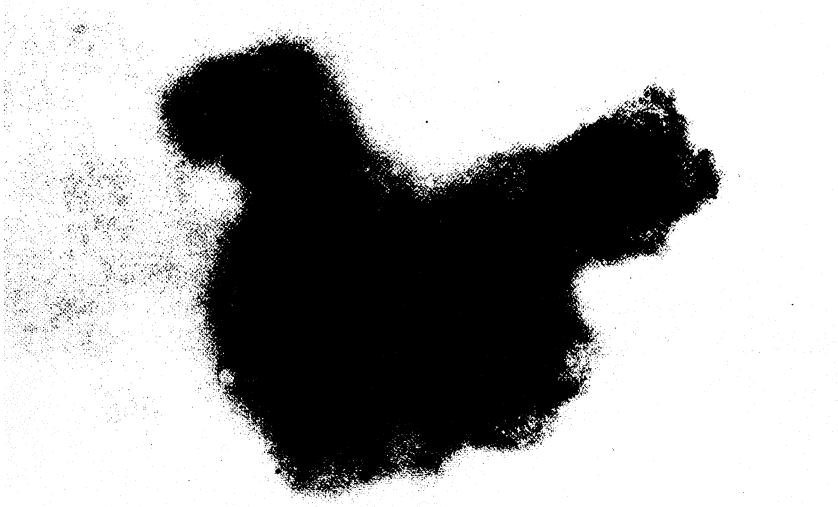

Fig. 2. Purified tissue-cultured equine rotavirus 576/84. The particles are aggregated by group specific immune serum, indicating that this equine rotavirus isolate belongs to group A of rotaviruses. (120,000 ×)

Table 2. Optical density obtained with a 1 : 10 dilution of indicated monoclonal antibodies when EqRv 576/84 was fixed to plates by polyclonal catching antibody

Subgroup I	Subgroup II	Common preparation
0.722	0.080	0.446

Numbers are arithmetric mean of the OD of two wells at 450 nm. Subgroup I = monoclone 255/60. Subgroup II = monoclone 631/9. Common preparation = a mixture of monoclones, designated as a broadly reacting agent.

Isolation of equine rotavirus in cell culture and partial characterization

Faecal samples were inoculated onto Ma 104 cells and the cells were observed for CPE daily. From day 7 p. i. virus was harvested and passaged. From one sample, an equine rotavirus could be isolated. Interestingly, in the faecal sample only a few rotavirus particles were detected, and they were not agglutinated by antiserum (Fig. 1). This strain — designated EqRv 576/84 — readily multiplied in Ma 104 cells, producing high titres of antigen when measured by ELISA.

After several sequential passages of EqRv 576/84 in Ma 104 cells, infectious titres of 10^7 $TCID_{50}$/ml were obtained.

The equine rotavirus was then propagated in greater quantities, concentrated by PEG-precipitation and finally purified by centrifugation into a preformed CsCl-gradient. Two bands could easily be detected and harvested. Their densitiy was 1.35 and 1.37 g/ml, respectively. They contained typical single-shelled and double-shelled rotaviral particles, respectively. Purified rotavirus preparation were easily agglutinated by group-specific antiserum (Fig. 2).

The subgroup antigen of EqRv 576/84 was assayed by ELISA using monoclonal antibodies. The results (Table 2) clearly show that this equine rotavirus has to be grouped into subgroup 1.

EqRv 576/84 failed to agglutinate erythrocytes of guinea pigs, rats or chickens.

Discussion

Over a period of four years epizootic outbreaks of diarrhoeal disease were observed in foals on a stud farm. We tried to evaluate the possible role of enteric viruses, namely rotavirus, in these cases. For virus demonstration, rotavirus ELISA, IEM and RNA-PAGE tests were used. In six out of 24 samples tested, the presence of rotavirus was detected by one or more assays. Similar observations on the frequency of rotavirus infections in foals have also been reported by GILLESPIE *et al.* (1984). These results clearly demonstrate that the possibility of rotaviral infections should always be taken into account when diarrhoeal disease is observed in foals.

The results of the group-specific ELISA, IEM and direct demonstration of viral nucleic acid by RNA-PAGE followed by silver staining were identical in 21 out of 24 samples tested. In two other samples only the ELISA or RNA-PAGE was positive. Sample 293/85 was positive by ELISA but negative by RNA-PAGE. RNA was probably lost during the purification procedure. On the other hand, sample 651/84 was negative by ELISA but positive by RNA-PAGE. Here, the antigenic sites which are recognized by ELISA were probably hidden by rotavirus antibodies. However, it cannot be excluded that this equine rotavirus is antigenically different from other animal rotaviruses.

In this study, no attempts were made to define the serotype of equine rotavirus. HOSHINO *et al.* (1983 a, b) reported the existence of two equine rotavirus serotypes from the USA. To date no other reports on the serological classification of equine rotaviruses are available, as only a few strains have been successfully propagated in tissue culture. Now that more cultivable equine rotavirus strains are available, all prerequisites are available for the development of prophylactic measures against neonatal diarrhoea in foals as is already the case for other animal species (EICHHORN *et al.*, 1983; SAIF *et al.*, 1984).

Zusammenfassung

Vorkommen und Isolierung in Zellkultur von equinen Rotaviren

Während eines Ausbruchs von Durchfallerkrankungen bei Fohlen eines Gestüts in Norddeutschland konnte bei sechs von 24 untersuchten Kotproben Rotavirus nachgewiesen werden. Aus einer Probe ließ sich equines Rotavirus in der Zellkultur isolieren. Dieses Isolat gehört zur Gruppe A der Rotaviren und innerhalb dieser Gruppe in die Untergruppe I.

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