RESEARCH ON BOVINE VIRAL DIARRHEA

Eiji TAKAHASHI*

Neonatal calf diarrhea: Rotavirus infection

Introduction

Epizootic diarrhea of new-born calves has for many years been the plague of stockbreeders. The disease attacks calves within four or five days of birth, causing at times a heavy mortality. Although some bacteria, *Escherichia coli* or *Salmonella* species, have sometimes been incriminated, it has been clear that they have not been the usual cause.

Mebus and his associates have demonstrated a reovirus-like agent to be the cause of the disease in the United States. Subsequent reports from various parts of the world have indicated a much wider distribution of this virus in the cattle population. In Japan, the infection was also been shown by serological tests to be widespread among cattle. In the present study we could isolate the virus in primary bovine kidney (BK) cell cultures from an affected calf in an outbreak of neonatal calf diarrhea. The name rotavirus has been proposed for this group of viruses. In the present report we will refer the virus as calf rotavirus.

We observed an outbreak of neonatal calf diarrhea at a breeding center in Shimane Prefecture over the period from January through March in 1977. Almost all the calves of Japanese black breed born at the center during this period were taken ill at one to 10 days of age with slight fever and watery yellowish diarrhea which severely dehydrated the calf. Of the 106 affected calves 44% died of dehydration.

Detection of virus particles in diarrheal feces

Diagnosis of rotavirus infection is usually based on the detection of virus or viral antigen in the feces.

Diarrheal feces and intestinal contents were collected from an affected calf (Shimane No.1) and prepared for demonstration of virus particles by electron microscopy. The mixture of feces and intestinal contents was homogenized to make a 10% suspension in PBS (0.15M NaCl, M/150 Phosphate buffer, pH7.2). The suspension was clarified by centrifugation at 3,000Xg for 20min and was centrifuged at 20,000Xg for 30min. The resulting supernatant fluid was successively filtered through membrane filters, 1,000, 800 and 650nm in pore size. The final filtrate was centrifuged at 100;000Xg for 2hr and the pellet was dissolved in 0.1 volume of PBS. The suspension was then centrifuged at 20,000Xg for 10min. Electron microscopic examination of the supernatant fluid by the phosphotungstic negative staining technique revealed numerous viral particles, 65-75nm in diameter, morphologically similar to the reovirus-like particles described by Mebus *et al.* and other workers.

Isolation of the virus

Attempts were made to isolate the virus from the feces and intestinal contents of the calf Shimane No.1. Primary BK cell cultures were prepared in 100× 11mm tubes. The growth medium was LE medium (Earle's solution containing 0.5% lactalbumin hydrolysate and 0.07% sodium bicarbonate) supplemented with 10% calf serum, and the maintenance medium was LE medium

* Senior Researcher, Virological Products Laboratory, Biological Products Research Division, National Institute of Animal Health.
Presently: FMD Diagnosis Laboratory, Second Research Division, National Institute of Animal Health, 1500 Jyosuihonmachi, Kodaira, Tokyo, 187, Japan.

containing 0.1% yeast extract. Cultures inoculated with 0.1ml volume, after virus adsorption at 37°C for 2hr, were washed three times with LE medium, fed with 0.5ml of maintenance medium, and incubated at 37°C in a roller drum. The cultures were checked for virus growth at each passage level by the direct immunofluorescence technique with antiserum to calf rotavirus. For the test the cultures were washed with PBS and scraped off into PBS with a rubber policeman. The cells were then spun down and smeared on glass slides with a micropipette. The preparations were air dried, fixed with acetone at −20°C for one hr, and stained at 37°C for one hr with fluorescein isothianate-conjugate. The antiserum used for the test was prepared in rabbits with the Lincoln strain of calf rotavirus as follows.

The virus was grown in primary BK cultures. Clarified culture fluid from infected cells was centrifuged at 100,000Xg for 2hr. The pellet was dissolved in 0.01 volume of PBS. The suspension was mixed with a CsCl solution to a density of 1.35g/ml and centrifuged in a Beckmann SW 50.1 rotor at 200,000Xg for 18hr. Infectivity and hemagglutinating activity peaked at the same density of 1.36g/ml and the peak fractions were used for immunization after two fold dilution of PBS. Each rabbit was inoculated intravenously with 5ml of the virus suspension. After 4 weeks, they were given a succession of two hyper-immunizing inoculations by the intramuscular route with one ml each of equal volume mixture of the virus suspension and Freund's complete adjuvant on two successive weeks. Serum was obtained 2 weeks later after the last inoculation. The conjugate was prepared as described by Mebus *et al.*

Virus isolation was tried three times and all trials were successful. In the first attempt the mixture of the feces and intestinal contents was homogenized to form a 10% suspension in PBS. The suspension, after centrifugation to remove coarse debris, was centrifuged at 20,000Xg for 30min and the supernatant fluid was used for inoculation into tube cultures of BK cells. Each of serial decimal dilutions, 10^{0} – 10^{-3}, of the material was inoculated into 10 BK culture. The inoculated cultures were incubated for 7 days and observed for any cytopathic effect (CPE) with negative results. At the end of the inoculation the cells from each group of tubes were pooled and tested for immunofluorescence. A few fluorescent cells were observed in the cultures inoculated with undiluted material. Further passages were made at intervals of 7 days with serial decimal dilution, 10^{0} – 10^{-3}, of pooled culture fluid from the tubes inoculated with undiluted material. Fluorescent cells were readily observed in all the cultures at the second and further passages, whereas CPE was first clearly noticed at the 7th passage. Further passages were readily accomplished. Immunofluorescence was first observed as perinuclear fine granules 3 days after the inoculation and became diffuse in the cytoplasm as the incubation progressed. The CPE appeared 2 to 3 days post-inoculation and was characterized by granulation, aggregation and eventual disintegration of the cells, leaving cell sheets of a moth-eaten appearance with many cells adhering to glass by the 5th day of incubation.

In the second trial of virus isolation the pooled specimen of feces and intestinal contents was processed in the same manner as in the first experiment and the supernatant fluid obtained after centrifugation at 20,000Xg for 30min was further treated by vigorously shaking with an equal volume of trichlorotrifluoroethane (chilled at −20°C). The material was then spun down in a refrigerated centrifuge at 3,000Xg for 20min and the supernatant fluid was used for inoculation into BK cell cultures.

In the third trial of virus isolation the intestinal contents from the calf were used. The material was processed as in the preceding experiments, and the supernatant fluid obtained after centrifugation (20,000Xg for 30min) was further centrifuged through discontinuous sucrose density gradients, 50, 30 and 10%, at 70,000Xg for 18hr. A band visible above the 50% layer was collected and centrifuged at 100,000Xg for 2hr. The pellet was dissolved in 0,01 volume of PBS and used for inoculation into BK cell cultures after centrifugation at 3,000Xg for 10min. Inoculation, passage and observation in the 2nd and 3rd trials were made in the same manner as in the first experiment. Cytopathic effect was clearly noticed at the 4th passage in the second trial and at the second passage in the third trial, respectively. Further passages were readily accomplished. The

demonstration of specific antigen of calf rotavirus in infected cells by the immunofluorescence staining indicated the isolated viruses to be calf rotavirus. This was further confirmed by the neutralisation test with the rabbit antiserum to calf rotavirus which was used for immunofluorescence staining.

More detailed comparison of the isolated viruses with the prototype Lincoln strain of calf rotavirus in needed for further confirmation of the identification of the isolated viruses.

Epizootic diarrhea of adult cattle caused by coronavirus-like agent

Introduction

The etiologic factors of bovine diarrhea are complex. Many viruses associated with this disease have been reported.

We observed outbreaks of epizootic diarrhea in cattle in more than ten areas in Japan during the winter of 1976 to 1977. Identical outbreaks of this disease have been observed subsequently. One hundred and fifty among 200 mixed-age cows in 10 herds from central and western parts of Japan developed profuse diarrhea and the milk production in these herds declined by 20-25% during the disease and only recovered to 80% of the previous production levels 3 or 4 weeks after the disease.

Serological evidence

One hundred paired sera from cattle with diarrhea were tested for hemagglutination-inhibition (HI) and neutralizing (NT) antibodies against bovine coronavirus (BCV), calf rotavirus (NCDV), bovine adenovirus type 7 (BAV-7), Parainfluenza virus type 3 (PIV-3) and bovine viral diarrhea virus (BVDV).

Seroconversion of HI antibody for BCV was detected in a high percentage (59%) of 100 paired sera tested, whereas a low percentage was detected for NCDV, BAV-7, PIV-3 and BVDV.

Very high incidence of seroconversion for BCV was also shown among cattle in 10 epizootic areas.

Detection of virus particles in diarrheal feces

Diarrheal feces were collected from an affected cow and prepared for demonstration of virus particles by electron microscopy. The feces were homogenized to make a 5% suspension in PBS. The suspension was clarified by centrifugation at 5,000Xg for 20min and was centrifuged at 20,000Xg for 30min. The resulting supernatant fluid was centrifuged at 100,000Xg for 2hr and the pellet was dissolved in 1/50 volume of PBS. The suspension was then centrifuged at 20,000Xg for 10min. Electron microscopic examination of the supernatant fluid by the phosphotungstic negative staining technique revealed numerous coronavirus-like agents, 60 to 120 nm in diameter.

Isolation of the virus

Attempts were made to isolate the virus from the feces of the cow (Shizuoka No. 16).

Primary BK cell cultures were prepared in 100 × 11mm tubes as described previously. BK cell cultures were inoculated with 0.1ml volume of purified materials from feces as described above. After virus adsorption at 37°C for 2hr they were washed three times with Earle's solution, fed with 0.5ml of maintenance medium and incubated at 37°C in a roller drum. The cultures were checked for virus growth at each passage level by the direct immunofluorescence technique with antiserum to bovine coronavirus supplied by Dr. C. A. Mebus. The antiserum and the conjugate were prepared by the method described above. Inoculation, passage and observation were made in the same manner as indicated previously.

A few fluorescent cells were observed in the cultures at the second passage level and many

distinct fluorescent cells were easily observed at the further passage levels. The CPE was recognized at the 8th passage of culture. The CPE appeared 3 to 4 days after the inoculation and was characterized by syncytium formation and granulation of the cells. As the passages increased, syncytia developed more quickly and were more distinct. The CPE was neutralized with the rabbit antiserum to bovine coronavirus which was used for immunofluorescence staining. Further identification of specific antigen of bovine coronavirus in infected cells was confirmed by the electron microscopic examination with the purified viral preparations obtained by sucrose gradient centrifugation. The negatively stained viral particles were similar to those detected in diarrheal feces from an affected cow.

Coronavirus-like agents have been associated with diarrhea in calves in the United States, Great Britain and Denmark. Horner and his associates have reported the observations of the agents in feces of cows with diarrhea in New Zealand. However, the etiological role of coronaviruses in the outbreak of diarrhea among the adult cattle had remained unknown. In the present report a coronavirus-like agent was isolated from the feces of a cow with diarrhea and this agent is morphologically and antigenically similar to the bovine coronavirus isolated in the United States. Serological surveys indicated a wide dissemination of coronavirus in the outbreak of diarrhea among the adult cattle in Japan. These observations suggest that the coronavirus-like agent is one of the principal causes of outbreak of diarrhea in adult animals. Further detailed comparison of the isolated virus with the bovine coronavirus in the United States will be necessary to confirm the identification and characterization of the isolated viruses.

Discussion

Gatapia, S.L. (Philippines): In the figures you presented, it appears that the cytopathic effect (CPE) is produced in the bovine kidney (BK) cells at the 7th passage, suggesting the need for a period of adaptation. May I know what was the interval between the passages?

Answer: Growth of virus is more active after the 7th passage. Usually the interval between passages is about one week. (7 days).

Horiuchi, T. (Japan): What is the simplest and most accurate method of diagnosis of corona virus infection?

Answer: Recently some immunological methods have been applied such as the ELISA test (enzyme-linked immunosorbent assay).

Kodama, M. (Japan): Does the virus replicate in the body of the cow?

Answer: I don't know.