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BOVINE ENTEROVIRUS BEV-T64

BIOLOGICAL CHARACTERISTICS. EXPERIMENTAL GENITAL AND DIGESTIVE TRACT INFECTION*

By

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The possible role of enteroviruses as etiological agents in reproductive disorders in cattle has received some attention during recent years. *Straub & Böhm* (1964) presented evidence that the agent causing catarrhal bovine vaginitis was an enterovirus. *Afshar et al.* (1964) isolated a bovine enterovirus from the vaginal mucus of a cow originating from a herd with a history of infertility and abortions. Only mild disorders were experienced following experimental infections. *Straub* (1965) isolated another enterovirus from the vagina of a cow with vaginal catarrh.

In the present study a virus was isolated in tissue culture from feces of a healthy 3-year-old cow. The biological characteristics identifying the agent as a bovine enterovirus and its ability to propagate in vaginal tissue and induce antibody formation is described in this paper.

MATERIALS AND METHODS

Virus source. The virus was originally isolated in primary monolayer tissue cultures of bovine fetal kidney grown in Earle's medium containing 0.5 % lactalbumin hydrolysate and 10 % inactivated calf serum. The third passage, propagated in primary bovine kidney tissue cultures with serum-free VM-3 medium (*Schwöbel & Siedentopf* 1961)

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served as virus stock for the experiments reported here. The virus stock was stored in 1 ml quantities in small vials at —65°C. The virus was designated BEV-T64.

Assay of virus infectivity. In those parts of the study where an assay of the virus infectivity was needed, the material to be tested was serially tenfold titrated in medium VM-3 containing 0.5 % lactalbumin hydrolysate and 0.2 % glucose. One ml from each dilution was inoculated into each of 3 tubes with primary bovine kidney tissue cultures and incubated at 37°C for 5 days. The cultures were then checked for presence of cytopathic effect, and the 50 % end point was calculated according to the method of *Kärber* (1931).

Virus propagation with various media. In this single test the first tissue culture passage served as virus source. The following media were tested for supporting the tissue cultures used for virus propagation:

1. Earle's medium containing 3 % calf serum
2. Earle's medium containing 3 % lamb serum
3. Earle's medium containing 1.5 % swine serum
4. Earle's medium with no serum
5. Tris medium containing 3 % calf serum
6. Medium VM-3 with 0.5 % lactalbumin hydrolysate and 0.2 % glucose
7. Bovine amnionic fluid

Ether and chloroform sensitivity. The tests were carried out according to standard procedures (*Andrewes & Horstmann* 1949).

Low pH-sensitivity. Equal volumes of stock virus and PBS with pH 7.2, 5.0 and 3.0 were mixed and divided into 2 equal parts; one was kept at room temperature for 1 hr. and the other was incubated at 37°C for 1 hr.

Temperature stability. The stability of the virus infectivity was tested by storage of virus samples at —20°C, 4°C, 20°C and 37°C for 8, 14, 25 and 31 days.

Heat sensitivity. Stabilization by cations. Stock virus was diluted 1:10 with PBS. To the first of 3 vials containing 5 ml each 5 ml of a 2 M-$MgCl_2$ solution, to the second and third another 5 ml of PBS were added. The first and second vials were then heated for 1 hr. in a water bath at 50°C, the third kept at room temperature.

Sedimentation constant. The test was carried out according to the method of *Strohmaier* (1966) using a sucrose gradient in an SW-25 rotor.

Virus propagation in chick embryos. Stock virus was serially tenfold titrated in PBS. The yolk sacs of 4-day-old embryos were inoculated with 0.25 ml from each dilution. Five days later the yolk sacs were harvested, homogenized, diluted 1:10 in PBS and centrifuged at 2,000 × g for 30 min.

Antiserum produced in chickens. Four 10-month-old chickens with no detectable serum neutralizing antibodies against the virus were injected intravenously (i.v.) 4 times at 2-day intervals with

1 ml of a virus suspension containing 1×10^6 TCID50 per ml. Each chicken received 4 ml i.v. 6 days later. The serum was harvested 12 days following the last injection.

Neutralization tests. A final virus concentration of 200 TCID50 per ml was tested against serial twofold dilutions of antiserum. The mixtures were kept at room temperature for 30 min. before the assay. The constant serum - diluted virus method was also used, the virus being serially tenfold titrated in medium containing 10 % antiserum. Preserum was used in the controls.

Animal experiments. Newborn, 3-day-old, and weanling mice were inoculated with stock virus. Sixteen animals, derived from 2 different litters, made up each group. Eight newborn mice were inoculated intraperitoneally (i.p.) with 0.1 ml virus suspension, and 8 animals were inoculated intracranially (i.c.) with 0.03 ml. Three mice in the 3-day age group were treated likewise. The mice at weanling age received 0.5 ml i.p. and 0.05 ml i.c. All inoculated mice were observed daily for a period of 3 weeks.

Four healthy heifers, 5 to 11 months old, that had no antibodies against the virus, were placed in an isolation unit. They were tied side by side, each animal having approximately 3 feet of space, and numbered 1 through 4, consecutively. Heifer no. 1 received 5 ml freshly harvested tissue culture virus suspension containing 1×10^6 TCID50 per ml intravaginally, and heifer no. 4 received the same dose intranasally. Heifers nos. 2 and 3 in between the inoculated animals did not receive any virus and served as contact controls. Body temperatures were taken daily and fecal samples as well as nasal and vaginal swabs were collected on days 1, 2, 3, 5, 10, 15, 19, 26, 44, 61 and 70. These samples were treated with antibiotics and bovine kidney tissue cultures inoculated as described earlier (*Straub & Böhm* 1962). Serum for neutralization tests was collected on days 24 and 50.

RESULTS

Virus propagation with various media. The TCID50 titers in tissue cultures with various media used, are shown in Table 1.

Table 1. Virus TCID50 titers in bovine kidney tissue cultures with various media.

Medium	TCID50 titer
1. Earle's medium containing 3 % calf serum	$10^{-1.5}$
2. Earle's medium containing 3 % lamb serum	$10^{-2.1}$
3. Earle's medium containing 1.5 % swine serum	$10^{-1.5}$
4. Earle's medium without serum	$10^{-2.5}$
5. Tris medium containing 3 % calf serum	$10^{-2.5}$
6. Medium VM-3 without serum	$10^{-3.8}$
7. Bovine amnionic fluid	$10^{-1.5}$

Ether and chloroform sensitivity. The virus proved to be resistant to the action of lipid solvents (Table 2).

Table 2. Virus TCID50 titers after treatment with lipid solvents.

	Treatment of virus		
	ether	chloroform	PBS (control)
TCID50 titer	$10^{-5.5}$	$10^{-6.2}$	$10^{-5.5}$

Low pH-sensitivity. The results of the test are recorded in Table 3.

Table 3. Virus TCID50 titers following incubation at various pH-levels.

	Treatment of virus	
pH	1 hr. at 20°C	1 hr. at 37°C
7.2	$10^{-4.8}$	$10^{-5.8}$
5.0	$10^{-5.2}$	$10^{-5.5}$
3.0	$10^{-5.5}$	$10^{-5.2}$

Temperature stability. The stability of the virus infectivity at different temperatures is demonstrated in Table 4.

Table 4. Virus TCID50 titers following storage at different temperatures.

Days of storage	Temperature			
	—20°C	4°C	20°C	37°C
8	NT*)	$10^{-6.2}$	$10^{-5.7}$	$10^{-5.2}$
14	$10^{-5.5}$	$10^{-5.7}$	$10^{-5.5}$	$10^{-4.2}$
25	$10^{-6.2}$	$10^{-6.2}$	$10^{-5.2}$	$10^{-3.2}$
31	$10^{-5.7}$	$10^{-5.7}$	$10^{-5.2}$	$10^{-2.5}$

*) NT = not tested.

Heat sensitivity. Stabilization by cations. The results are recorded in Table 5.

Table 5. TCID50 virus titers following exposure to heat in the presence and absence of 1 M-$MgCl_2$.

	Treatment of virus		
	PBS 50°C 1 hr.	1 M-$MgCl_2$ 50°C 1 hr.	PBS 20°C 1 hr.
TCID50 virus titer following treatment	$10^{-4.8}$	$10^{-4.5}$	$10^{-4.5}$

Sedimentation constant. A sedimentation constant between 140 and 150 S was recorded.

Virus propagation in chick embryos. Inoculation of virus into embryonated eggs caused deaths of 2 out of 3 embryos in the dilutions 10^{-1} and 10^{-2}. No deaths were recorded in the higher dilutions. The virus infectivity titers of harvested yolk sac material from the dead embryos were $10^{2.8}$ and $10^{3.0}$ higher than in the original inoculum, respectively.

Antiserum produced in chickens. The final serum dilution of 1:2048 caused a 50 % neutralization of 200 TCID50 of the virus. A neutralization index of 5.6 was recorded employing the constant serum - diluted virus technique.

Animal experiments. No signs of disease occurred in any of the mice inoculated.

The inoculated heifers showed no increase in body temperature nor any other signs of illness during the observation period.

Table 6. Recovery of virus after experimental inoculations of heifers. Animal no. 1 had been inoculated intravaginally, no. 4 intranasally, and nos. 2 and 3 served as contact controls.

Animal no.	Source of sample for virus recovery	Days post inoculation for sampling										
		1	2	3	5	10	15	19	26	44	61	70
1	nose	—	—	—	—	—	—	NT	NT	NT	NT	NT
	vagina	+	—	+	+	+	—	NT	NT	NT	NT	NT
	feces	—	—	—	—	+	+	+	—	—	—	—
2	nose	—	—	—	—	—	—	NT	NT	NT	NT	NT
	vagina	—	—	—	—	—	—	NT	NT	NT	NT	NT
	feces	—	—	—	—	+	+	+	+	+	—	—
3	nose	—	—	—	—	—	—	NT	NT	NT	NT	NT
	vagina	—	—	—	—	—	—	NT	NT	NT	NT	NT
	feces	—	—	+	+	+	+	+	+	+	+	+
4	nose	—	—	—	—	—	—	NT	NT	NT	NT	NT
	vagina	—	—	—	—	—	—	NT	NT	NT	NT	NT
	feces	+	+	+	+	+	+	+	—	+	—	—

\+ = virus recovered.
— = no virus recovered.
NT = not tested.

The results of the attempts to recover virus and those of the serum neutralization tests of the inoculated animals are summarized in Table 6 and Fig. 1.

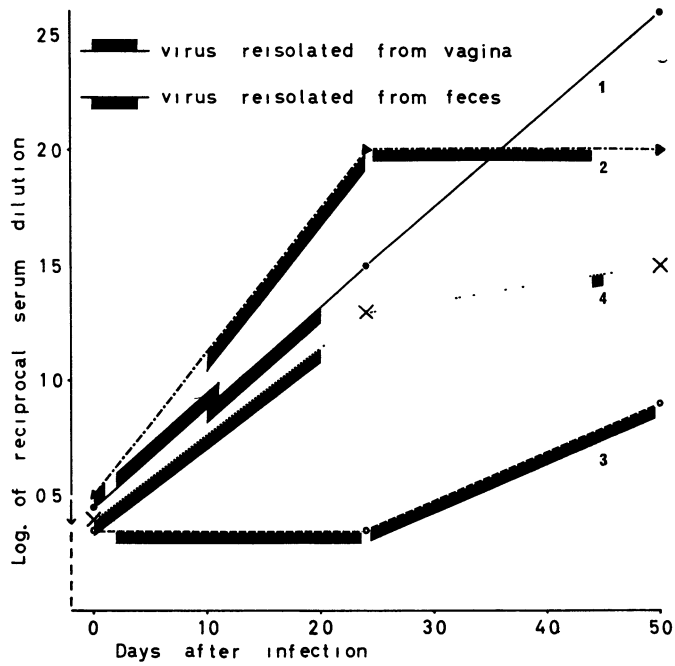

Figure 1. Serum neutralizing antibodies to BEV-T64 virus in experimentally infected heifers. 1: infected intravaginally. 2 and 3: contact controls. 4: infected intranasally. The values are recorded as the log. of the reciprocal serum dilution causing 50 % neutralization of 200 TCID50 virus.

DISCUSSION

The BEV-T64 virus, originally isolated from the feces of a healthy cow, has the characteristics of a bovine enterovirus. It is resistant to lipid solvents and to pH 3. A remarkable stability was observed following exposure to high temperature (50°C) for 1 hr. and to storage at 37°C for a prolonged period of time. No pathogenicity to mice could be demonstrated, whereas the virus replicated and caused deaths in chicken embryos.

The experimentally induced infections in heifers make up the most interesting part of the present study. The experiments are limited, but some interesting speculations can be made from

the results obtained. Animal no. 4 was intranasally inoculated and started to shed the virus in the feces the next day. Virus was reisolated from fecal samples up to day 20. A positive sample was also recorded on day 44. The infection was followed by production of serum neutralizing antibodies reaching a titer of log 1.5 50 days after the infection. Animal no. 3, stabled next to no. 4, started the excretion of virus on day 3 and continued throughout the observation period. The level of neutralizing antibodies remained very low between days 24 and 50.

Animal no. 1 was intravaginally infected. Virus was reisolated from the vaginal mucosa on the day following the inoculation. On day 2 virus recovery test was negative, but positive from day 3 throughout day 10. Virus could be reisolated from feces from day 10 throughout day 19. The highest levels of neutralizing antibodies were recorded in this animal.

Animal no. 2, next to no. 1, excreted the virus in feces from day 10 till day 44 in spite of a relatively high level of neutralizing antibodies present in the serum.

In animal no. 1 the virus was obviously propagated in the vaginal tissue. It is remarkable that this genital infection induced a higher production of circulating antibodies than did the infection in the animals infected by the nasal or oral route. Lymphocytes and plasma cells are consistently found in the mucous membranes of the digestive (*Crabbé et al.* 1965) and genital (*Skjerven* 1956) tracts. There is evidence that antibody production can occur within the alimentary canal, the genital tract and the mammary gland (*Lascelles et al.* 1966, *Pierce* 1959). The observations made in the present study indicate that the genital infection induced a higher production of circulating antibodies than did the digestive tract infection.

The presence of circulating antibodies apparently inhibited a prolonged replication of virus in the vaginal mucosa, while the same, or higher titer of antibodies, was unable to prevent a propagation in the alimentary canal. The possible role of locally excreted antibodies and viral tissue tropism should, however, be considered in this connection.

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SUMMARY

A virus isolated from a healthy cow was tested for biological characteristics. It was resistant to lipid solvents, stable at pH 3 and to 50°C for 1 hr. and showed a remarkable stability during storage at various temperature levels. The virus replicated and caused deaths in chicken embryos, whereas no pathogenicity for mice was recorded. Chickens immunized developed high-levels of humoral antibodies to the virus. A sedimentation constant between 140 and 150 S was recorded. The agent possesses the characteristics of an enterovirus and is designated BEV-T64.

Limited studies on experimental infections in heifers indicated that the animal genitally infected developed higher levels of circulating antibodies than those infected intranasally or orally (by contact). The shedding of virus from the genital tract terminated after 10 days, whereas virus was reisolated for as long as 44 and 70 days in cases of alimentary canal infection. No clinical signs of disease could be observed.

SAMMENDRAG

Bovint enterovirus BEV-T64.

Biologiske egenskaper. Eksperimentell genital og alimentær infeksjon.

Et virus opprinnelig isolert fra ei klinisk frisk ku ble testet med hensyn på klassifisering. Det var resistent mot fettløsningsmidler og stabilt ved pH 3. Infeksjonstiteret ble ikke påvirket av varmebehandling (50°C) i 1 time, og viruset var bemerkelsesverdig stabilt ved lengre tids lagring ved ulike temperaturer. Virus replikerte i kyllingfostre og viste her patogenitet, mens podning på mus ga negative resultater. Ved å bruke fjørfe ved immuniseringen fikk man fram et antiserum med høyt titer. Sedimentasjonskonstanten lå mellom 140 og 150 S. Dette agens har egenskaper som tillegges enterovirusgruppen og får betegnelsen BEV-T64.

Ved begrensede eksperimentelle studier på kviger syntes en genital infeksjon å forårsake sterkere dannelse av sirkulerende antistoffer enn den alimentære infeksjon. Virusutskillelsen fra genitaltraktus opphørte etter 10 dager, mens virus ble reisolert fra feces opptil 44 og 70 dager fra dyr som hadde alimentær infeksjon. Kliniske symptomer ble ikke observert.

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