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Isolation and cultivation of bovine ephemeral fever virus in chickens and chicken embryos

By M. A. GAFFAR ELAMIN* and P. B. SPRADBROW
Department of Veterinary Pathology and Public Health, University of Queensland, St Lucia, Brisbane, Australia

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SUMMARY

Unadapted bovine ephemeral fever (BEF) virus was isolated from cattle blood after intravenous inoculation into chicken embryos. Infected embryos died or hatched as abnormal chickens. The chick embryo was slightly less sensitive to unadapted BEF virus than were Vero cell cultures, but the use of embryos avoids the several blind passages that are required to isolate BEF virus in unweaned mice. Chick embryos were considerably less efficient than Vero cell culture or unweaned mice in detecting Vero cell-adapted and mouse-adapted BEF virus respectively.

Viraemia was demonstrated in chicken embryos at 1–4 days and in one-day-old chickens at 1–3 days after intravenous inoculation of BEF virus. BEF virus was demonstrated by isolation and by immunofluoresence in heart, brain, lung and liver of chicken embryos at 1–5 days and in lung and liver of one-day-old chickens at 1–2 days, after intravenous inoculation. The isolated viruses were confirmed as BEF virus by neutralization with immune mouse ascitic fluid. BEF neutralizing antibodies were produced in 4-week-old and adult chickens after intravenous inoculation with BEF virus.

INTRODUCTION

Experimental inoculation of cattle with bovine ephemeral fever virus has shown that intravenous inoculation is the only route by which the virus can be regularly transmitted and that 0·01 ml of infective blood was capable of initiating infection in cattle (Mackerras, Mackerras & Burnet, 1940). These findings, together with the epizootiological considerations, suggested that BEF virus was an arbovirus. Since then experiments designed to investigate the role of the insect vectors and the biological transmission of the causative agent have been unsuccessful. However, there have recently been isolations of BEF virus from insects in Kenya (Davies & Walker, 1974) and in Australia (St George, Standfast & Dyce, 1976).

The growth of modified strains of BEF virus in chicken embryos was described by Tzipori & Spradbrow (1974). The present communication describes the isolation of unmodified BEF virus from cattle blood in chicken embryos, provides further

* On study leave from Veterinary Research Laboratory, Khartoum, Sudan.

information on experimental infection of chicken embryos and explores the susceptibility of hatched chickens to BEF virus.

MATERIALS AND METHODS

Source of viruses

Unadapted BEF virus was obtained from bovine blood. Blood was collected from calves 130, 133, 175 and 186 during the clinical reaction to experimentally induced BEF, and from a cow (Mog 1) which contracted BEF during a natural outbreak.

Various laboratory strains originating from strain 919 or strain 525 (Tzipori, 1975*a*) were kindly made available by Dr S. Tzipori. They comprised a strain adapted to Vero cell cultures (919 V_{12}), a strain adapted to unweaned mice (525 MB_3) and this mouse-adapted strain after passage in chick embryo and Vero cells (525 MB_3 $CE_{10}V_{14}$).

Inoculation of chicken embryos and chickens

The technique for intravenous inoculation of 10–13-day-old chicken embryos was that described by Boorman, Mellor, Penn & Jennings (1975). Twenty-five 1-day-old chickens were inoculated intracardially or into the jugular vein with volumes of 0·3–0·5 ml.

Virus assay

Virus assay was conducted in Vero cells or in unweaned mice as described by Snowdon (1970) and the titres were expressed as 50% tissue culture infectious doses (TCID 50) or 50% lethal doses (LD 50). BEF virus was also assayed in chicken embryos, using death of embryos as the end point, and the embryo LD 50 was calculated by the method of Reed & Muench (1938).

Neutralization tests

Neutralization tests on chicken sera were performed by a technique similar to that described by Snowdon (1970). Serial twofold dilutions of heat-inactivated serum (56 °C for 30 min) were mixed with an equal volume of cell culture fluid containing 100 TCID 50 of BEF virus. After incubation for 60 min at 37 °C each mixture of virus and serum was used to inoculate at least four tube cultures of Vero cells. The highest serum dilution preventing cytopathic change in at least half the culture was taken as the endpoint. A titration of the test virus was included in each assay.

Isolation of BEF virus from cattle blood in chicken embryos

Chicken embryos were inoculated intravenously with 0·02 ml volumes of whole blood, plasma and 20% suspensions of buffy coat cells in phosphate buffered saline from calves 130 and 186. The embryos were candled daily and surviving embryos were allowed to hatch. Deaths occurring within 24 h of inoculation were

regarded as non-specific. Attempts were made to recover BEF virus from the brain of dead embryos and hatched chickens by intracerebral inoculation of suckling mice, three blind passages being performed before samples were considered negative.

Distribution of BEF virus in organs of infected chicken embryos and 1-day-old chickens

Twenty-five chicken embryos 10 or 11 days of age were inoculated intravenously with the $133MB_8$ strain of BEF virus containing $10^{6.0}$ mouse LD50/ml. In the first trial three embryos were killed each day and pooled blood and brain were tested for the presence of BEF virus by the intracerebral inoculation of suckling mice. The brains of normal and abnormal chickens hatching from these eggs were similarly tested.

In a second trial, three embryos were destroyed each day and heart, brain, lung and liver were harvested. A portion of each organ was used to prepare a 20% suspension which was titrated in unweaned mice. Another portion of each organ was used to prepare frozen sections which were fixed in acetone and stained with fluorescein-conjugated BEF virus immune mouse ascitic fluid as described by Murphy, Taylor, Mims & Whitfield (1972). Control preparations consisted of sections from normal chicken embryos and fluorescein conjugated globulin from normal mouse ascitic fluid.

Similar trials were conducted in 1-day-old chickens inoculated intravenously with the same preparation of BEF virus.

Comparison of the sensitivities of chicken embryos, Vero cell culture and unweaned mice to various strains of BEF virus

The following preparations were titrated in chicken embryos, unweaned mice and Vero cell cultures – $133MB_8$, $525MB_3$, $919V_{12}$ and whole blood from calves 130 and 175 and cow Mog 1.

Effect of length of incubation on the susceptibility of chicken embryos to BEF virus

A preparation of strain $133MB_8$ BEF virus was titrated in chicken embryos that had been incubated for 10 days and 13 days respectively before inoculation.

The serological response to BEF virus in chickens of various ages

Eleven 4-weeks-old and six adult fowls were inoculated intravenously with 0·5–1·0 ml of a preparation of BEF virus strain $525MB_3\,CE_{10}V_{14}$ containing $10^{5.0}$ TCID50/ml. Two chickens from each group were bled daily for 6 days after inoculation, and the blood was tested for viraemia by intracerebral inoculation of unweaned mice. All chickens were bled at weekly intervals for 6 weeks and serum neutralizing antibodies were titrated.

Table 1. *Distribution of bovine ephemeral fever virus in organs of chick embryos and chickens after intravenous inoculation with* 133 MB_8 *strain of bovine ephemeral fever virus*

		Heart		Brain		Lung		Liver	
Host	Days after inoculation	Titre	Degree of fluorescence	Titre	Degree of fluorescence	Titre	Degree of fluorescence	Titre	Degree of fluorescence
Chicken embryos	1	0	1+	0	0	0	1+	0	1+
	2	1·2	2+	1·3	2+	1·5	2+	1·5	2+
	3	1·5	2+	1·5	3+	1·5	2+	1·5	3+
	4	0	1+	1·5	3+	1·2	2+	1·5	2+
	5	0	–	1·5	2+	0	–	–	1+
1-day-old chickens	1	0	–	0	–	0	2+	0	1+
	2	0	–	0	–	NT	2+	0	–
	3	0	–	0	–	NT	1+	NT	–
	4	0	–	0	–	NT	–	0	–
	5	0	–	0	–	NT	–	0	–

0, No virus isolated.
Degree of fluorescence: –, no fluorescence; 3+, maximum; 2+, medium; 1+, minimum.
Titre, LD50/0·01 ml of homogenate.
NT, Not tested.

RESULTS

Isolation of BEF virus from infected cattle blood in chicken embryos

BEF virus was recovered from whole blood, plasma and buffy coat of infected calves 130 and 186. Most chicken embryos were found dead in shell late in the incubation period, or chickens hatched with abnormalities similar to those described by Tzipori & Spradbrow (1974). A few hatched chickens appeared clinically normal. After hatching, the virus could be recovered from the brains of clinically abnormal as well as normal chickens. The virus was isolated in unweaned mice and 2–3 blind passages were required before regular mortalities were produced. The virus recovered from chickens was neutralized by BEF virus immune mouse ascitic fluid.

Distribution of BEF virus in organs of infected chicken embryos and 1-day-old chickens

The results are shown in Table 1. Viral antigen was detected in liver, lung and heart of the infected chicken embryos during the early stages of infection and a little later in the brain. The same patterns were seen with virus isolated from these organs. No virus was isolated from any organ of the infected 1-day-old chickens, while fluorescence was demonstrated in the lung. Fluorescent antibody control preparations failed to show fluorescence.

Infected chicken embryos were viraemic from the first to the fourth days after inoculation. BEF virus viraemia was detected in 1-day-old chickens 1–3 days after inoculation. The viruses were isolated from the viraemic 1-day-old chickens in

Table 2. *Comparative sensitivity to bovine ephemeral fever virus of chicken embryos, unweaned mice and Vero cell cultures*

Virus strain	Log_{10} LD50/ml (chick embryos)	Log_{10} LD50/ml (mice)	Log_{10} TCID50/ml (Vero culture)
133 MB_8	3·9	7·0	6·5
525 MB_3	3·5	5·9	5·0
919 V_{12}	2·5	–	5·2
Mog 1 blood	2·5	–	3·0
175 blood	2·0	–	3·0
130 blood	2·5	–	3·0

–, Blind passage was needed for adaptation of the virus to mice.

Table 3. *Neutralizing antibody response against bovine ephemeral fever virus in 4-week-old and adult chickens after intravenous inoculation with chicken-adapted strain of BEF virus* (525 $MB_3C_{10}V_{14}$)

Age	No. of chick-ens	Mean neutralizing antibody titre (weeks after inoculation) 1	2	3	4	5	6
4 weeks	11	1·6	2·0	1·8	1·8	< 1	< 1
Adult	6	1·7	2·7	3·0	2·3	1·3	1·3

unweaned mice after 2–3 passages, and viruses were confirmed as BEF virus by neutralization with BEF mouse ascitic fluid.

A comparison of the sensitivities of chicken embryos, cell culture and unweaned mice to BEF virus

The results are shown in Table 2. Assay of BEF in chicken embryos, as judged by death of embryos, was less sensitive than assay in Vero cell culture. Unweaned mice were more sensitive than chicken embryos for mouse-adapted virus.

Effect of length of incubation on the susceptibility of chicken embryos to BEF virus

Chicken embryos inoculated with strain 133 MB_8 at the incubation age of 10 days were found more susceptible (titre $10^{3·8}$ egg LD50/0·1 ml) than those inoculated at the incubation age of 13 days (titre $10^{2·9}$ egg LD50/0·1 ml).

BEF neutralizing antibody in chickens of various age groups

The results are shown in Table 3. Nine of eleven 4-week-old chickens and five of six adult birds developed low titre neutralizing antibodies against BEF virus after intravenous inoculation. The peak titre did not exceed 4. No viraemia was detected.

DISCUSSION

In the present study intravenous inoculation of chicken embryos was found insensitive for BEF virus assay when compared with Vero cell culture or unweaned mice. In part, this was due to the survival of some chickens which were

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harbouring BEF virus, the embryo infectious dose being higher than the embryo lethal dose. However, chick embryos would be useful for the isolation of unadapted virus from cattle because results are obtained on the first passage, in contrast to the several passages needed to adapt BEF virus to unweaned mice. Hitherto only cattle and chick embryos were known to respond with viraemia after intravenous inoculation with BEF virus. It is now known that BEF virus will also produce viraemia in hatched chickens and this allows the speculation that birds may be susceptible to infections in nature.

Tzipori & Spradbrow (1974) were able to recover BEF virus from the brain of chicken embryos infected intravenously. In the present study both isolation and immunofluorescent tests were used to demonstrate viral persistence in lung, liver, heart and brain of infected chicken embryos. The distribution of BEF virus in experimentally infected chicken embryos is similar to that in experimentally infected cattle (Kodama, Sasaki, Kikuyama & Ishii, 1973).

The fluorescent antibody technique for the study of BEF virus was first used by Theodoridis (1969) and then by Murphy *et al.* (1972). In the present study immunofluorescence offered an alternative system to the cumbersome isolation methods usually employed to demonstrate BEF virus. The response to infection in the organs of chicken embryos could be demonstrated by fluorescent antibody staining. A possible explanation of this sequence is that the virus multiplies in the vascular endothelium before it is transferred to target cells in the brain. Thus the finding of the virus in liver, heart and lung might indicate trapping of the virus rather than replication.

The development of neutralizing antibodies in 4-week-old and adult chickens was probably an indication of virus multiplication. However, the proposed replication might be restricted and undetectable by the routine procedures. On the other hand, the development of viraemia in chicken embryos inoculated intravenously and the lesser viraemia in 1-day-old chickens could be indicative of an inverse age susceptibility in chickens.

It is known that, except with some unusual strains of BEF virus (Tzipori & Spradbrow, 1974), younger mice are more susceptible than older mice. Although young calves are often regarded as insusceptible to BEF, Tzipori (1975*b*) produced severe clinical disease, viraemia and long lasting neutralizing antibodies in colostrum-deprived newborn calves, inoculated intravenously with BEF virus. An increased susceptibility of young animals is a feature of many arbovirus infections.

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