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STUDIES ON BOVINE EPHEMERAL FEVER. I. ISOLATION AND PRELIMINARY CHARACTERIZATION OF A VIRUS FROM NATURAL AND EXPERIMENTALLY PRODUCED CASES OF BOVINE EPHEMERAL FEVER

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INTRODUCTION

Ephemeral fever or three-day-stiffsickness of cattle has been known for nearly a century, and has been described in East Africa (Schweinfurth, 1867), Rhodesia (Bevan, 1907), South Africa (Theiler, 1908), Indonesia (Merkins, 1919; Burggraaf, 1932), India (Meadows, 1919), Japan (Futamara, 1922), Palestine (Rosen, 1931), and Australia (Mulhearn, 1937). During recent years world-wide surveys on the incidence of ephemeral fever, published in the FAO, WHO and OIE animal health yearbooks (1957–1965), have shown that this disease has a wide geographic distribution in Africa and Asia.

Bevan (1912) reported natural and experimental transmission of the disease to sheep. This observation was subsequently confirmed by Futamara in Japan, who claimed to have transmitted the disease not only to cattle and sheep, but also to rabbits and mice.

Mackerras, Mackerras & Burnet (1940) carried out extensive clinical and pathological studies on the experimentally produced disease in Australia. They succeeded in maintaining the virus through 80 serial passages in cattle and demonstrated that in blood the infectivity is associated exclusively with the leucocyte-platelet fraction. They failed to transmit the disease to horses, sheep, goats, dogs, rabbits, guinea-pigs, rats or mice, and concluded that the virus is host specific, and almost certainly transmitted by insects.

In South Africa ephemeral fever has a seasonal incidence and manifests itself during late summer and autumn as sporadic outbreaks of a febrile disease, which terminate soon after the first frosts in May. Some observations on the clinical symptoms and the economic importance of the disease in dairy herds in South Africa were published by MacFarlane & Haig (1955).

The object of this paper is to record the isolation and preliminary characterization of strains of virus from naturally and experimentally produced cases of the disease in cattle.

MATERIALS AND METHODS

(*a*) *Infective material*

A sample of citrated whole blood, collected at the height of the febrile reaction from an experimentally produced case of ephemeral fever was used to commence this study. The original blood sample, collected in 1958, had been stored in sealed ampoules under dry ice refrigeration during the intervening period of six years.

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A two-year-old steer, maintained in an insectproof stable, was inoculated intravenously with 3·0 ml of the original stored blood sample. This animal was kept under observation, and the rectal temperature recorded at four-hourly intervals. At the height of the febrile reaction blood was collected into 1·5 litre flasks containing 150 ml of a 10 per cent w/v solution of sodium citrate. At the same time blood smears were made, stained with Giemsa and examined microscopically to exclude possible intercurrent infections of anaplasmosis and babesiosis.

The blood was transferred into 250 ml bottles and centrifuged at 2700 rpm for 30 minutes. Thereafter the supernatant plasma was decanted and discarded, while the leucocyte-platelet fraction was removed through a No. 14 gauge needle (without a bevel) attached to a 10 ml syringe.

The cell fractions were pooled, washed twice and resuspended in saline to give a final concentration of 1:5. The infectivity of this cell suspension was determined by inoculating 3·0 ml of the material into an ox and then observing the ensuing reaction. This infective material was stored at −76° C and used as stock challenge virus.

(*b*) *Experimental animals*

(i) Cattle between the ages of nine and 72 months were used, without regard to sex or breed. These animals, consisting mostly of Friesian, Jersey and Afrikaner cross-breeds, were either reared on the experimental farm adjoining the Institute or acquired from other parts of the country.

(ii) Litters of unweaned albino mice between the age of one and three days were obtained from the closed colony maintained at the Institute. Before inoculation the suckling mice were randomized and groups of seven allocated to each female.

(*c*) *Serial passage and assay of virus in mice*

Infant mice *in extremis* were killed with ether and their brains removed aseptically. A 10 per cent brain suspension prepared in sterile saline was centrifuged at 3000 r.p.m. for 10 minutes. The supernatant was used for the further inoculation of mice by the intracerebral route in a volume of 0·03 ml.

Infectivity titres were determined by the inoculation of groups of mice with serial ten-fold dilutions of mouse brain suspension. End points were calculated by the method of Reed & Muench (1938).

(*d*) *Serological tests*

Serum samples were collected from each bovine immediately before inoculation and again three to four weeks after the termination of the febrile reaction. The serum was Seitz filtered and stored at −18° C. Immediately before inclusion in a neutralization test, each sample was inactivated at 56° C for 30 minutes.

(i) Serum-virus neutralization test: The 9th serial mouse brain passage of the first strain of virus isolated was used in these tests. The virus was stored in a dry ice cabinet as a 10 per cent mouse brain suspension in a 0·2 M phosphate buffer solution containing 5 per cent lactose and 1 per cent peptone at a pH of 7·4.

Quantitative serum-virus neutralization tests were set up by mixing equal volumes of undiluted serum with serial ten-fold dilutions of virus. After incubation at 37° C for 45 minutes, 0·03 ml of each serum-virus mixture was injected intracerebrally into each of a group of suckling mice. Mortality was recorded at daily intervals during a period of three weeks observation.

(ii) Complement fixation tests: In the complement fixation tests, predetermined standard amounts of antigen, two units of complement, an optimal dilution of amboceptor and two-fold dilutions of serum were used.

Antigens for bluetongue, Rift Valley fever and Wesselsbron viruses were prepared from infected suckling mouse brains by acetone-ether extration (Clarke & Casals, 1958).

Known specific immune and negative control sera for each antigen were included in the tests.

The complement fixing antigen for the virus under investigation was prepared from the 9th serial intracerebral mouse passage of the first virus isolate, and consisted of a 20 per cent suspension of infected mouse brains in saline, clarified by centrifugation at 3,000 r.p.m. for 10 minutes.

(*e*) *Assessment of the severity of clinical reactions in experimental cattle*

In an attempt to obtain some degree of uniformity in the assessment of the severity of the clinical reactions encountered in experimental cattle from one experiment to another, a scale representing four levels of clinical involvement was drawn up. These data were compiled from accumulated experimental records and observations of the naturally occurring disease within the enzootic area. A summary of the most prominent symptoms and their relation to the course of the disease is given in Table 1. The abbreviated scale is used where necessary in successive tables.

TABLE 1.—*Assessment of the severity of clinical reactions in experimental cattle*

Designated scale	Distinguishing symptoms
+	Fever of short duration and inappetence
+ +	Fever, inappetence slight shivering, nasal and/or eye discharges, slight stiffness
+ + +	Fever, inappetence, nasal and eye discharges, shivering moderate stiffness with dejection
+ + + +	Fever, inappetence, nasal and eye discharges, shivering with severe lameness and stiffness, sometimes paretic, usually recumbent. Utter dejection

RESULTS

(*a*) *Isolation of the prototype virus EF I*

The leucocyte-platelet fraction, representing the stock challenge virus obtained from the original stored blood sample, was inoculated into suckling mice.

The mortality recorded in the first and subsequent intracerebral passages is given in Table 2.

TABLE 2.—*Mortality and mean survival time in suckling mice with serial passage of virus EF I*

	Passage level in suckling mice									
	1	2	3	4	5	6	7	8	9	10
Percentage mortality.....	17	62	100	100	100	100	100	100	100	100
Mean survival time (days).	12·4	8·3	5·0	4·8	4·6	4·1	3·6	2·8	2·8	2·8

In the first passage a few mice from different families showed signs of lethargy and paralysis commencing on the 10th day after inoculation. After the 3rd passage 100 per cent mortality of the inoculated mice was observed but it was not until the 8th serial passage that the survival time was reduced to a constant figure. No mortality was recorded in sucklings or older mice weighing 15 gm and inoculated by the intraperitoneal route with various passage levels of the virus.

(*b*) *Action of sodium desoxycholate (DCA) on virus isolate EF I*

The inactivation of arboviruses by sodium desoxycholate under controlled conditions was described by Theiler (1957), as a preliminary test in the classification of viruses pathogenic for suckling mice.

The technique used by Theiler was followed closely. Infected suckling mouse brain in the form of a 10 per cent w/v suspension in saline containing 0·75 per cent bovine albumin, was clarified by centrifugation at 10,000 r.p.m. for one hour in a high speed angle head of a refrigerated centrifuge. The supernatant fluid was distributed in two duplicate sets of two tubes each. To each of the two tubes of the one set an equal volume of bovine albumin-saline was added, and to the two tubes of the other set an equal volume of 1:500 sodium desoxycholate. Both tubes were then incubated at 37° C. One tube from each set was removed after half an hour and the other after one hour.

The results given in Table 3 show that the virus is sensitive to the action of DCA.

TABLE 3.—*The action of sodium desoxycholate (DCA) on virus EF I*

Virus	Period of incubation at 37° C	Virus LD_{50}		Difference
		DCA	Control	
EF I (9th i.c. passage level).....	½ hour	1·6*	4·3	2·7
	1 hour	1·0	3·6	2·6

* Logarithmic index of the reciprocal of the end point dilution as determined in suckling mice

(*c*) *Action of diethyl ether*

A virus suspension was prepared as previously described. To a sample of this suspension diethyl ether was added to give a final concentration of 20 per cent. To a duplicate control sample an equivalent volume of bovine albumin-saline was added instead of ether. Both samples were incubated in screw capped bottles at 4° C for 18 hours. After removal of the ether from the one sample by evaporation, the infectivity of the two samples was determined by titration in suckling mice.

These results are shown in Table 4 and clearly indicate that the virus is inactivated in the presence of 20 per cent diethyl ether.

TABLE 4.—*Action of diethyl ether on virus EF I*

Virus	Virus LD_{50}		Difference
	Ether	Control	
EF I (9th i.c. passage level).....	0·4*	4·0	3·6

* Logarithmic index of the reciprocal of the end point dilution as determined in suckling mice

(*d*) *Preliminary identification by complement fixation*

The occurrence of bluetongue amongst clinically healthy cattle in the area, where these experiments were conducted, has been described by Du Toit (1962). The possible occurrence of Rift Valley fever (R.V.F.) and Wesselsbron virus in cattle within the endemic ephemeral fever areas prompted the application of the group specific complement fixation test as a means of eliminating these pathogens as contaminants.

The results of these tests are given in Table 5 where it will be observed that there was no fixation between the virus EF I and the antisera to bluetongue, R.V.F. or Wesselsbron virus.

TABLE 5.—*Complement fixation with virus EF I*

Antigen	Dilution of antigen	Antiserum		
		Blue-tongue	Rift Valley fever	Wessels-bron virus
Virus EF I.....	Undiluted	0	0	0
	1:2	0	0	0
Normal mouse brain.....	Undiluted	0	0	0
	1:2	0	0	0
Bluetongue.....	1:32	48*	—	—
Rift Valley fever.....	1:32	—	32	—
Wesselsbron.....	1:32	—	—	24
Normal mouse brain.....	1:4	0	0	4

* Reciprocal of the serum dilution giving 50 per cent fixation of complement

(e) Inoculation of cattle with EF I virus

Six head of cattle with no detectable neutralizing antibodies to virus EF I in their sera were selected for this experiment. Immediately prior to inoculation the animals were again bled for serum.

Two animals were injected, one subcutaneously and the other intravenously, with 5 ml of a 10 per cent w/v infective suckling mouse brain suspension representing 10,000 mouse LD_{50} of the 3rd serial passage of virus EF I. Two other animals were similarly inoculated, one subcutaneously, the other intravenously with the 9th serial passage of the virus. The two remaining animals served as controls. The animals were kept under observation and their temperatures recorded every six hours. Four weeks after inoculation, they were bled for serum and then each injected with 3·0 ml of stock challenge virus.

As shown in Table 6 there was no clinical or febrile response, and the animals failed to develop antibodies following the injection of the mouse adapted virus. It, therefore, appeared that this virus was not pathogenic to cattle.

TABLE 6.—*Clinical and antibody response of cattle inoculated with virus EF I, 3rd and 9th serial passages*

Bovine No.	Passage level and route of inoculation of virus EF I	Clinical response	Rise in antibody	Virulent blood challenge	
				Clinical reaction	Neutralization index after challenge
1	3rd passage subcutaneously.....	None	None*	+ + + + *	10,000†
2	3rd passage intravenously.....	None	None	+ + + +	9,031
3	9th passage subcutaneously.....	None	None	+ + + +	10,000
4	9th passage intravenously.....	None	None	+ + +	10,000
5	Control.....	None	—	+ + + +	10,000
6	Control.....	None	—	+ + +	10,000

* See Table 1 for severity scale of symptoms

† Expressed as the log mouse LD_{50} neutralized

These results led to the following experiment.

Five susceptible cattle were selected and each was injected subcutaneously with 10 ml of inoculum consisting of an equal amount of a 10 per cent suspension of infective suckling mouse brain of the 9th passage level of virus EF I (approximately 10,000 mouse LD_{50}) and adjuvant (1 part Arlacel A and 9 parts Bayol F). Three such injections were given at weekly intervals. Two control animals were given a similar series of injections with normal mouse brain suspension in adjuvant. Fourteen days after the last injection, the animals were bled for serum and each inoculated with 3·0 ml of stock challenge virus.

As can be seen in Table 7, the five animals which received repeated injections of infective mouse brain suspension developed antibodies against the virus, while the controls, inoculated with normal mouse brain suspension, failed to develop detectable antibodies. After challenge the four animals, whose sera showed high neutralizing indices, failed to react. One animal, whose serum revealed a low neutralizing index showed a moderate reaction after challenge whereas the two controls reacted severely and showed marked symptoms of the disease.

TABLE 7.—*Development of antibodies in cattle, repeatedly inoculated with virus EF I, and subsequent challenge with virulent ephemeral fever blood*

Bovine No.	Virus LD_{50} in 1:2 dilution of serum		Neutralization index	Virulent blood challenge 3cc I/V
	Prebleed	Postbleed after 3 inoculations		Clinical reaction
2412	4·8†	0†	63100	No reaction
2174	4·2	2·3	79	+ +*
3474	4·4	0·6	6310	No reaction
1309	4·5	0·3	15850	No reaction
2508	4·0	0·3	5012	No reaction
Controls	—	—	—	—
3705	4·5	4·5	0	+ + + +*
3476	4·3	4·3	0	+ + + +

* See Table 1 for severity scale of symptoms

† Logarithmic index of the reciprocal of the endpoint dilution as determined in suckling mice, expressed as the mouse LD_{50} of virus neutralized

(*f*) *Detection of specific neutralization antibodies against virus EF I in experimentally and naturally infected cattle*

(i) *Cattle experimentally inoculated with ephemeral fever virus:* A group of 21 head of cattle, was selected at random from a pool of cattle available for experimental purposes.

After collection of serum samples, each animal was injected intravenously with 3·0 ml of the stock challenge virus.

The inoculated animals were observed daily, and their rectal temperatures recorded at six-hourly intervals. After 30 days, serum samples were again collected for neutralization tests.

The clinical reactions, together with the results of the serum-virus neutralization tests, conducted on the paired serum samples from these animals are given in Table 8.

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TABLE 8.—*Reaction and antibody rise in cattle experimentally infected with ephemeral fever blood*

Bovine		Incubation period (days)	Severity of clinical symptoms	LD_{50} of virus EF I in 1:2 dilution of serum		Neutralization index
No.	Age (approx.) (years)			Pre-infection	Post-infection	
2075	4	—	No reaction	0·3†	0†	2
1311	6	—	No reaction	0·2	0·2	0
2080	4	—	No reaction	0	0	0
1308	6	—	No reaction	0·6	0·4	2
3765	1	—	No reaction	0·7	0	5
2074	4	—	No reaction	0·1	0·1	0
2330	1·5	4	+ + +*	4·1	0·7	2512
2324	1·5	6	+ + + +	4·0	0·7	1995
2251	1·5	6	+ + + +	4·0	0·3	5012
2077	4	5	+ +	2·1	0·3	63
2374	1·5	4	+ + +	4·2	0·2	10000
2057	2	5	+ + + +	4·1	0·1	10000
2659	4	6	+ + +	4·0	0·6	2512
2353	2	6	+ + +	3·2	0	1585
2071	1·5	5	+ + +	4·6	0·4	15850
2662	0·75	5	+ + +	3·9	0·3	3981
3678	2	6	+ + + +	2·4	0·2	158
3697	2	6	+ + + +	3·8	0·6	1585
3685	2	6	+ + +	4·0	0	10000
3665	2	6	+ + + +	4·2	0·4	6310
2929	4	4	+ + + +	4·2	0·6	3981

* See materials and methods for scale of clinical symptoms

† Logarithmic index of the reciprocal of the endpoint dilution as determined in suckling mice, expressed as the mouse LD_{50} of virus neutralized

Six of the animals, whose sera showed evidence of neutralizing antibodies to virus EF I prior to the injection of the challenge virus failed to react. The remaining fifteen animals, with no EF I virus neutralizing antibodies in their sera prior to the experiment all reacted and showed typical symptoms of ephemeral fever. The serum samples collected 30 days after challenge showed a rise in their neutralizing antibody titre to virus EF I.

(ii) *Detection of antibodies in the sera of recovered animals after natural infection:* During the late summer of 1965, a group of approximately 200 cattle, comprising mainly Afrikaner-Hereford crossbreeds, held under ranching conditions, was kept under observation in an attempt to determine the prevalence of natural infection. These animals, supervised by reliable stockmen, were bled for serum as soon as clinical symptoms were observed and again three weeks later. It is considered unlikely that animals with mild symptoms would have been observed under these conditions, consequently only animals with severe lameness and stiffness were reported.

Using virus EF I as antigen, serum-virus neutralization tests were conducted on the paired serum samples. The severity of the disease in the affected cattle and the development of antibodies are recorded in Table 9.

TABLE 9.—*Development of antibodies in naturally infected cases of ephemeral fever*

Bovine No.	Date symptoms first observed	Severity of clinical symptoms	LD_{50} of virus EF I in 1:2 dilution of serum		Neutralization index
			Acute phase	Convalescent phase	
2023	27/1/65	+ + + +*	4·2†	0†	15850‡
1129	27/1/65	+ + + +	3·0	0	1000
2199	15/2/65	+ + + +	3·6	0	3981
1128	22/2/65	+ + + +	3·2	0	1585
2386	22/2/65	+ + + +	1·5	0·3	16
2359	22/2/65	+ + + +	4·5	0	31620
2276	23/2/65	+ + + +	3·0	0	1000
1607	23/2/65	+ + + +	4·5	0	31260
2254	23/2/65	+ + + +	3·7	0	5012
3018	22/3/65	+ + + +	3·2	0	1585
1306	23/3/65	+ + + +	4·6	0	39810

* See materials and methods for assessment of the severity of the clinical reaction
† Logarithmic index of the reciprocal of the endpoint dilution as determined in suckling mice
‡ Expressed as the mouse LD_{50} of virus neutralized

Eleven of the animals exposed to natural infection developed symptoms of disease diagnosed clinically as ephemeral fever. The acute phase serum samples from these samples failed to reveal neutralizing antibodies against the mouse adapted virus EF I, whereas the convalescent phase serum samples all revealed high neutralizing indices against this virus.

(iii) *Further isolation of virus from field specimens:* Twelve blood samples, collected from natural cases of the disease in the northern and western Transvaal and north-western and eastern Cape Provinces of South Africa, were inoculated intravenously into susceptible cattle. When these animals reacted, with typical symptoms of ephemeral fever, they were bled and the leucocyte-platelet fraction prepared as previously described. The isolation procedure described for virus EF I was followed with these samples.

The successful recovery in mice of four additional virus isolates, designated EF III, IV, VI and IX, is recorded in Table 10. Their neutralization by specific antiserum against virus EF I is given in Table 11.

TABLE 10.—*Mortality and mean survival time in suckling mice in first and subsequent passage levels of additional field isolates*

Virus Isolate No.		Passage level in suckling mice						
		1	2	3	4	5	6	7
EF III	Per cent mort*.....	9·6	74	100	100	100	100	100
	M.S.T. (days)†.....	10·8	9·2	6·1	5·6	5·6	4·3	4·0
EF IV	Per cent mort.....	11·2	89·7	100	100	100	—	—
	M.S.T. (days).....	14·8	7·5	6·6	5·3	5·1	—	—
EF VI	Per cent mort.....	7·3	61·1	79·4	100	100	—	—
	M.S.T. (days).....	14·6	11·2	8·7	7·3	6·4	—	—
EF IX	Per cent mort.....	14·1	81·1	100	100	—	—	—
	M.S.T. (days).....	12·1	8·6	5·2	4·7	—	—	—

* Per cent mortality
† Mean survival time (days)

TABLE 11.—*Neutralization of additional field virus isolates by virus EF I convalescen serum*

Neutralization test antigen	i.c. passage level in mice	LD_{50} of virus in 1:2 dilution of serum		Neutralization index
		Pre-infection	Virus EF I Convalescent	
Virus EF I.....	9	4·6*	0	39810†
Virus EF III.....	7	3·7	0	5012
Virus EF IV.....	5	3·5	0	3162
Virus EF VI.....	5	3·8	0·7	1259
Virus EF IX.....	4	2·7	0	501

* Logarithmic index of the reciprocal of the endpoint dilution as determined in suckling mice
† Expressed as the mouse LD_{50} of virus neutralized

DISCUSSION

This paper records the isolation of a virus in mice from the leucocyte-platelet fraction of whole blood of a bovine suffering from ephemeral fever. Subsequently, this was confirmed by the isolation of four additional strains of virus from the blood of cattle experimentally infected with field samples. All these strains were neutralized by the convalescent phase serum of a bovine infected with the original virus thus showing that they are immunologically similar.

The injection of cattle with the 3rd and 9th serial passages of the mouse adapted virus EF I failed to produce either clinical evidence of disease or a specific immunological response. However, after repeated inoculations of the virus together with an adjuvant, antibodies comparable to those found in animals recovered from natural attacks of ephemeral fever developed.

The extremely rapid modification and the loss of pathogenicity of the virus for cattle is surprising, but a similar observation has been made with the adaptation of dengue fever virus in mice. According to Sabin (1952) the Hawaiian strain lost its capacity to produce severe illness and fever in humans as early as the 7th passage in suckling mice but in contradistinction to the modified ephemeral fever virus, it retained its immunogenic properties. Further work is necessary to determine whether other strains of ephemeral fever virus will behave in a similar manner.

The loss of pathogenicity of the mouse-adapted virus for cattle has precluded the fulfilment of Kock's postulates, and the direct identification of the virus as the cause of ephemeral fever. However, sufficient indirect evidence has been presented to support the conclusion that this virus is the cause of the clinical syndrome recognized as bovine ephemeral fever in South Africa.

It has been shown that cattle with naturally acquired antibodies to the virus were refractory to challenge with virulent ephemeral fever virus, while those without significant antibody titres were susceptible to challenge. The modified virus only stimulates antibody production after repeated inoculations before it protects cattle against challenge with virulent virus. Furthermore it has been conclusively demonstrated that cattle recovering from an experimental or natural infection, develop a high neutralizing antibody titre in their convalescent sera. The neutralization test would thus appear to be satisfactory for the selection of animals for experimental purposes, and the study of different strains of ephemeral fever virus.

The observations of Mackerras, Mackerras & Burnet (1940), as well as those made on the epidemiology of ephemeral fever in South Africa strongly suggest that the disease is insect-borne. The additional evidence of the virucidal effect of DCA and ether on the infectivity of the virus appears to strengthen this supposition that ephemeral fever virus belongs to the arboviruses, although further studies are necessary before this classification can be confirmed.

Summary

Five immunologically identical strains of virus have been isolated from naturally and experimentally produced cases of ephemeral fever, by the intracerebral inoculation of suckling mice with infective buffy coat suspension.

The prototype strain was found to be DCA and ether sensitive. Specific neutralizing antibodies could be demonstrated in the convalescent sera of both naturally and experimentally infected cattle.

Serial passage of the virus in suckling mice resulted in a very rapid loss of pathogenicity for cattle with a corresponding loss of immunogenicity.

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