Aust. J. Biol. Sci., 1987, **40**, 137–41

Preliminary Studies of the Complement Fixation Test to Confirm the Diagnosis of Bovine Ephemeral Fever

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Abstract

A strain of bovine ephemeral fever (BEF) virus isolated in China in 1976 was adapted to growth in tissue cultures. A baby hamster kidney complement fixing (CF) antigen, stable at −20°C for at least 120 days, was prepared from the BEF virus grown in tissue culture and used to test bovine sera for antibodies to that virus. CF antibodies were detected in all of 31 cattle after convalescence from experimental infection with BEF virus, in 208 (98%) of 213 cattle observed to have shown clinical ephemeral fever in an epidemic, in 96 cattle in these herds which did not show clinical signs of ephemeral fever and 16 cattle from herds in northern China outside the epidemic area. The CF antibodies to BEF virus were found to persist in 34 (89%) of 38 cattle which were bled 6 years after natural exposure to ephemeral fever. The CF antigen is economical to prepare and is suitable to differentiate ephemeral fever from other viral infections with which it could possibly be confused on clinical appearance.

Extra keywords: infectious bovine rhinotracheitis virus, bovine respiratory syncytial virus, parainfluenza 3 virus, bovine rhinovirus.

Introduction

Bovine ephemeral fever (BEF) is an acute, febrile, viral disease of cattle and buffalo. Since the disease was described in Africa in the 19th Century (Piot 1896), it has been reported from many countries in Oceania and Asia (St George 1981). This probably records recognition rather than spread. BEF was first recognised in China in the 1950's (Bai Wenbin, unpublished data) and there have been several epidemics in recent years.

It is often difficult to distinguish clinically mild BEF from other viral diseases of cattle such as infectious bovine rhinotracheitis, bovine respiratory syncytial virus infection, bluetongue-like disease, parainfluenza type 3 virus infection, bovine adenovirus infection and bovine rhinovirus infection. Therefore, researchers of many countries have placed emphasis on confirming a diagnosis of BEF by specific serology. In recent years, we have studied the possibility of the application of the complement fixation test for the serological confirmation of BEF virus infection.

Materials and Methods

Virus

A virus strain (Beijing 1) isolated from a naturally infected case of ephemeral fever bled 20 September 1976 at Beijing, China, has been adapted to growth in BHK21 cell cultures. This was the virus strain used to prepare the antigen. This strain of virus has since been compared with the Australian prototype strain of BEF virus and shown to be similar (Tian Fenglan *et al.* 1987).

0004-9417/87/020137$02.00

Antigen Preparation

The virus strain was grown in monolayers of BHK21 cells. The growth medium consisted of equal volumes of medium 199 and medium RPMI 1640 (GIBCO, U.S.A.) plus 10% (v/v) inactivated fetal calf serum. The maintenance medium was similar but contained only 2% (v/v) inactivated fetal calf serum. The pH of the media was adjusted to 7 with 5·6% (w/v) $NaHCO_3$. Sufficient penicillin and streptomycin were added to give a final concentration of 100 units of penicillin and 100 mg/ml of streptomycin.

The cell cultures were grown for 48–72 h in glass bottles. The supernatant culture fluid was then decanted and the virus at a dilution of 1 : 100 was added to the bottles, and absorbed for 1 h at 37°C. The inoculum was discarded and the original volume of maintenance medium added. The tissues were incubated at 34°C for 48–72 h. When more than 90% of the cells showed cytopathic effect, the cells were scraped from the glass. These harvested cells were then frozen and thawed three times at −20° and 37°C respectively. The suspensions were poured into flasks, to which guinea pig serum, inactivated at 56°C for 30 min, was added to give a final concentration of 2% (v/v). The suspension was then allowed to stand overnight at 4°C. The following day, the suspensions were again frozen and thawed five times at −20° and 37°C respectively, then centrifuged at 2500 rpm for 20 min. The resultant supernatant was collected and centrifuged at 20 000 rpm for 1 h. The supernatants were collected and used as the virus antigen (V antigen). Control antigen (C antigen) was prepared from uninfected BHK21 cells subjected to the same procedures.

Preparation of Sensitized Sheep Red Blood Cells

Washed sheep red blood cells (3% v/v) were mixed with an equal volume of 2 units of titrated haemolysin (Chungdou, China) and the mixture allowed to stand for 30 min at room temperature and then for 10 min at 37°C in a water-bath to sensitize the sheep red blood cells.

Preparation of the Reference-positive Serum

Pre-bled cattle were injected intravenously seven times with virulent BEF virus which had been stored as defibrinated blood. The interval between the first and second injection was 2 weeks. Five subsequent injections were followed at weekly intervals. The doses of blood were 10, 10, 15, 20, 25, 30 and 35 ml respectively. Serum was collected 10–15 days after the last injection, and titrated. When antibody titres were satisfactory the cattle were bled to provide a stock of serum. Otherwise another booster injection was given. Sera were stored at −20°C until used.

Antigen Titration

Using the reference-positive serum, the antigen preparations were titrated by complement dilution procedure. A marked difference existed between the titres of V and C antigen preparations when the reference-positive serum was used, whereas no difference existed when physiological saline and negative serum were substituted.

Complement Titration

Sera were collected from 5 to 10 male guinea pigs (>450 g), pooled and stored at −20°C until used as complement. Complement titration procedures were as follows: complement diluted 1 : 10 with physiological saline was added to No. 1 of a row of tubes, then serially diluted (7 volumes of complement to 3 of physiological saline) to Nos 10–12. The dilutions of complement were mixed thoroughly with the constant C antigen, negative serum and sensitized sheep red blood cells. The complement titres were read after incubation for 30 min at 37°C in a water-bath which determined accurately the dilution used in the formal test.

Complement Fixing Test Procedures and Reading Standards

Serum diluted at 1 : 4 and appropriate dilutions of complement and antigen were mixed by stirring vigorously. The tubes were allowed to stand for 16–20 h at 0–4°C. After 30 min at room temperature, the sensitized sheep red blood cells were added, mixed thoroughly and incubated together for 30 min at 37°C. The results were read after the unhaemolysed red blood cells had sedimented for 2–4 h at room temperature. The reading was made on the basis of difference of haemolysis inhibition between the V and C antigen rows. The reading standard for BEF was as follows: sera with a titre greater than 0·3 were given as positive, 0·1–0·2 as uncertain and 0 as negative.

V and C antigen rows were read in pairs and compared with adjacent tubes to avoid errors. Only when the differences existed between more than two pairs of tubes was there a reading made. If no differences existed between one pair, the reading was rated as doubtful and the test was repeated. The conventional controls were applied to each day's complement fixing (CF) test.

Testing of Serum and Antigen Stability

Sera collected from experimentally infected, naturally infected and healthy cattle, as well as sera from cattle infected with other diseases, were inactivated for 30 min at 56°C and stored at −20°C until used. The prepared antigen was stored at −20°C with regular titrations.

The CF test was used to test the specificity of the prepared antigen for the convalescent-phase (21–30 days after recovery) serum from 31 experimentally infected and 213 naturally infected cattle. Eighty sera from cattle in herds in which BEF had occurred, but which had not shown clinical signs, were also tested. Sera from 16 cattle in herds in BEF-free areas of northern China were also tested in order to detect non-specific reactions. The possibility of cross-reactions produced by other virus infections were tested for using the prepared antigen and sera from cattle infected with infectious bovine rhinotracheitis (IBR), bovine virus diarrhoea (mucosal disease), bovine leucosis and theileriosis.

Table 1. Results of examination of the convalescent-phase sera from cattle naturally infected with BEF in various parts of China

Source	No. of cattle	Results	
		Doubtful	Positive
Lutai	15	1	14
Shoukou	4	–	4
Zhengzhou	14	–	14
Wuhan	5	1	4
Changsha	22	1	21
Yantang	5	–	5
Xinzhou	19	1	18
Hangshou	25	–	25
Nanjing	5	–	5
Hefei	20	–	20
Jinan	20	–	20
Beijing	7	–	7
Shanghai	30	1	29
Dalian	22	–	22
Total	213	5	208 (98%)

Results

V Antigen Titration

Using the reference-positive sera, five batches of V antigen were assessed in the CF test. The results showed that the titres of four batches were in the range of 1·5–1·9 and one batch was in the range 1·2–1·4, whereas with negative serum and physiological saline the titres of all batches were 0. As the titre of antigen was required to exceed 1·5 for practical use, four of five batches of V antigen proved satisfactory.

Storage of V Antigen

Two batches of V antigen (the initial titres of which were 1·5), stored at −20°C for 60 and 120 days respectively, were titrated without any loss of CF activity.

Examination of Serum

The convalescent-phase sera of 31 experimentally infected cattle were positive in the CF test. The results of CF tests of convalescent-phase serum from 213 natural cases of BEF, which originated from 15 areas in 1983, are shown in Table 1. All 16 sera collected from BEF-free herds plus the 80 sera from cattle which had not shown clinical BEF during an epidemic were negative in the CF test.

In order to test further the specificity of the V antigen preparations, tests were made with two known IBR-positive sera, nine sera from cattle positive to IBR, three to bovine virus diarrhoea, 46 to bovine leucosis and 12 to theileriosis. All these sera proved negative.

The persistence of antibodies in the convalescent-phase serum in cattle was determined by examining sera from cattle with BEF which occurred in 1976 in Beijing, Xian, and Guangzhou (Table 2); 34 (89%) of 38 sera were still positive.

Table 2. Results of the retrospective examination of serum antibodies from 38 cattle naturally infected with BEF 6 years previously

All cattle infected August–September 1986; all sera collected August 1982

Source	No. of cattle	Results		
		Negative	Doubtful	Positive
Beijing	29	–	2	27
Xian	5	1	–	4
Guangzhuo	4	1	–	3
Total	38	2	2	34

Discussion

The results obtained in this study showed that 100% of the convalescent-phase sera from cattle experimentally infected with BEF and 97·8% of those sera from the animals naturally infected with BEF were positive for CF antibodies. The positive titres persisted at least 6 years after the last-known natural infection with BEF.

Sera from healthy herds, with and without a history of BEF occurrence, were negative. In addition, the sera from cattle with IBR, bovine virus diarrhoea and theileriosis did not cross-react with the V antigen. All tests demonstrated clearly that the V antigen obtained by freezing and thawing had a high specificity. Kishi (1968) prepared CF antigens from the brains of unweaned hamsters and albino mice injected with BEF virus using the method of Casals *et al.* (1967). This antigen provided a specific test with sera from cattle which had recovered from natural or experimental infection with ephemeral fever. However, the antigen preparation from mouse or hamster brains was difficult to use on a large scale because of low yields combined with a more difficult preparation procedure.

Ito *et al.* (1969) reported the use of antigens prepared from the brains of suckling mice, suckling hamster, suckling rats and BHK21 cells for CF tests for ephemeral fever, but the latter yielded a low-titre antigen. The results obtained with the V antigen prepared by freezing and thawing from BHK21 cell cultures infected with the Beijing strain of BEF virus was not only specific, but also had the advantage

of being simple to obtain on a large scale. However, the titre of this antigen can probably be further improved.

The V antigen stored for 60 and 120 days at −20°C proved to be stable but no systematic comparative tests were carried out to determine the optimum storage conditions or the effects on titre of storage at various temperatures.

This test has been shown to be of practical value for confirming natural or experimental ephemeral fever infections. The fact that the antigen is stable on storage means that it can be held at the central laboratory or distributed to provincial laboratories for use as the situation requires. The central production of the antigen and antisera eliminates most of the test standardization problems.

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Manuscript received 9 September 1986, accepted 4 February 1987