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Bovine Ephemeral Fever and Related Rhabdoviruses

Proceedings of the 1st International Symposium held in Beijing, PRC, 25–27 August 1992

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Contents

Taxonomy, Morphology and Molecular Biology 75

Vaccines and Antigenic Variation 109

Appendices

Foreword

A massive epidemic of ephemeral fever in China in 1983 led to a request to Australia in 1984 for a joint research program on the epidemiology and control of the disease to be sponsored by the Chinese Academy of Agricultural Sciences and ACIAR. A meeting was held at the Harbin Veterinary Research Institute in December 1984 to discuss the feasibility of the proposal. This brief two-day meeting between Chinese and Australian scientists, with enormous communication difficulties, through an interpreter without experience of scientific terminology, blossomed into effective cooperative research and free interchange of information. Australia had much more to offer on ephemeral fever because of a long experience with the disease but the flow of knowledge soon became both ways. The official end to the involvement of ACIAR in ephemeral fever is marked by this symposium. However, the friendships developed by scientists during visits and shared adventures will continue. The names of the Chinese and CSIRO scientific team leaders and members, the project supervisors from the Chinese Ministry of Agriculture, Academy of Agricultural Sciences and ACIAR, are listed in these Proceedings. However, without the support of the parent organisations and general goodwill, the scientific interchange would have been impossible.

It would have taken a vivid imagination to predict that the meeting in the snow and ice of a Harbin winter would lead to the first international symposium on ephemeral fever being held this summer in Beijing. Many of the problems of ephemeral fever remain, but major advances have been made and this symposium will serve to bring them to the notice of the world.

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Summary of Research Needs

The symposium has produced a wealth of information on ephemeral fever particularly in China and demonstrated that active research is proceeding. Some of the aspects of research which need attention are shared by China with other areas of the world.

- Ephemeral fever is emerging as a more serious economic disease in Asian and African countries. Its characteristic of producing more serious disease in higher producing animals is thus highlighted as breeding and nutrition improve.
- There is a need to evolve cheaper anti-inflammatory regimes to counter the clinical signs of ephemeral fever in less developed countries which cannot afford the effective, but expensive, non-steroidal anti-inflammatory drugs. The effective technique evolved in some Chinese provinces of immersing water buffalo almost completely in water to reduce fever should be made widely known.
- The central collation of statistics on ephemeral fever occurrence, morbidity and mortality would show that it is a very serious economic disease. The low mortality has meant that the loss of milk, muscle destruction and disablement of draught animals at ploughing and harvest, is not appreciated. The monetary loss needs objective assessment. Office International des Epizooties should again collate its occurrence in the world.
- The presently available commercial vaccines are not adequate for prevention or control in terms of efficacy and price. The new subunit, cell culture derived vaccines undergoing field trials are effective but need to be produced cost effectively by modified concentration techniques, and by recombinant technology.
- The papers presented at this symposium show clearly that the antigenicity of bovine ephemeral fever (BEF) virus is not lost on passage through mice or tissue culture. The subunit vaccines derived from Chinese (Beijing 1) and Australian (BB7721) isolates are fully antigenic and induce immunity in cattle. The inability of passaged BEF virus to induce disease which has been linked in the past to antigenic loss may be due to a diminished efficiency to replicate in cattle and produce a sufficient mass of antigen.
- Water buffalo are infected during ephemeral fever epidemics, though the prevalence tends to be lower. There is a need for an objective description of the disease in buffalo; the isolation of BEF virus from buffalo blood; preservation of infected buffalo blood for transmission to cattle and buffalo; and comparison at the molecular level with BEF virus of cattle origin.
- The study of disease produced by BEF-related viruses such as Puchong and Kotonkan is in its infancy. As vaccines against ephemeral fever are highly unlikely to be effective against infection with these viruses, differential diagnosis is important in countries where related viruses exist.

Criteria for diagnosis of ephemeral fever:

For over a century, the diagnosis of ephemeral fever has rested on the recognition of the characteristic history in a herd or population basis, in particular the sudden onset and often abrupt recovery from severe disease. Several stages of disease are present together. It was recognised by the symposium that many countries lack the resources to diagnose the disease, when it appears at irregular intervals, or in an unusually severe form.

A provisional diagnosis can be made on history and a rise in antibody titres from negative to 16 or greater, between sera taken during illness and 14 days from recovery.

The transmissibility of the disease by transfer of febrile blood intravenously will distinguish ephemeral fever from non-infectious diseases which share some clinical signs.

A definitive diagnosis requires the isolation of virus from blood taken early in illness for isolation in *Aedes albopictus* tissue cultures, BHK21 cell cultures, or intracerebrally in suckling mice. The virus should then be identified by neutralisation by polyclonal and monoclonal antisera to BEF virus, to distinguish it from related rhabdoviruses.

It is recognised that complement fixation and neutralisation tests are useful, but may give false positives in areas where BEF related viruses exist. The competitive ELISA test using monoclonal antibodies is the most specific test developed so far and should be made more widely available.

There is a need for international reference laboratories. These could operate on a regional basis. However, representative stocks of BEF virus should be collected, with good histories of origin, so that antigenic variation can be monitored. This variation is important for epidemiology and to ensure that vaccines contain appropriate antigens.

Epidemiology and Vectors

The early accounts of ephemeral fever in southern Africa suggested that the disease could be insect-spread. The pattern of major epidemics, which are well documented as being independent of cattle movements and influenced by weather, support the involvement of flying insects.

The direct evidence for insect vector involvement is sparse. Bovine ephemeral fever (BEF) virus has been isolated four times from different *Culicoides* species associated with cattle: three times in Africa; and once in Australia. Isolations have also been made from mosquitoes of both the *Anopheles* and *Culicines* genera. These isolations indicate that BEF virus is readily taken up by haematophagous insects. The presence of a virus within an insect does not necessarily mean that the insect can transmit the disease. It is known, however, that BEF virus is readily transmittable from a viraemic animal to a susceptible animal if infective blood is delivered directly into a vein. This is how mosquitoes feed but not *Culicoides*. Also, like ephemeral fever, the response of mosquito populations to rain and river height fluctuations is rapid whereas *Culicoides* increase their population density in response to such climatic changes more slowly. The epidemiological evidence from Australia also shows that mosquitoes are the most important vectors and research efforts should be directed towards them.

Several papers were presented at the symposium on the epidemiology of ephemeral fever in China. There are additional papers in the companion volume in Chinese. The published papers, however, only touch on the extent of the detailed and very accurate records that have been kept at the local level in China. The opportunity therefore exists for a retrospective study to correlate this vast disease record with climatic factors in China over the same period. Prospective studies are also needed to determine what species of mosquito bite cattle as a guide to confirmatory virus isolation and transmission studies.

The Natural History of Ephemeral Fever of Cattle

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Abstract

Ephemeral fever is caused by an insect-borne rhabdovirus which is widely distributed through Africa, Asia and Australia. Based on epidemiology, and the necessity for bovine ephemeral fever (BEF) virus to be injected into a vein for disease transmission, mosquitoes of various *Anophelene* or *Culicine* species are likely to be more efficient vectors of the virus than the *Culicoides* species from which BEF virus has been isolated. The definitive vectors have yet to be identified.

Ephemeral fever disease in cattle is associated with a generalised inflammation and toxaemia, possibly due to massive interferon production stimulated by BEF virus. A superimposed hypocalcaemia causes short term paralysis which can be reversed by infusion of calcium salts. The clinical signs due to inflammation can be completely prevented or resolved by the administration of anti-inflammatory drugs. The biochemical spectrum of changes in plasma zinc, iron, copper, calcium, glucose, inorganic phosphate and fibrinogen are non-specific characteristics of inflammatory diseases. There are also high plasma ammonia (NH_3) levels and non-esterified fatty acid levels during clinical disease plus elevated pH.

BEF virus probably multiplies entirely within the vascular system. The virus has been detected in plasma and neutrophils in the bloodstream and neutrophils in tissues, probably as a result of phagocytosis. The virus has not been detected in lymphocytes or erythrocytes. Natural infection of cattle appears to result in life-long immunity with no evidence of latent infection.

THE first scientific account of ephemeral fever was written under the title *'Epizootic dengue fever of cattle'* (Piot 1896). An epidemic of ephemeral fever swept from south to north in Egypt the previous year, affecting a third of the cattle population. Piot was struck by the similarities of the clinical signs in cattle to those of dengue in man, also a disabling disease but usually not fatal. The next scientific account of the clinical signs was by Bevan (1907), but it was Freer (1910) who, in describing the disease as 'ephemeral fever' mentioned two key points, the requirement for intravenous inoculation of blood from a clinically ill cow to transmit the disease to a susceptible cow, and that the epidemiology suggested spread by biting midges. The disease was recognised subsequently in many countries of Africa and Asia including Australia where it first appeared as a massive epidemic in 1936–37 (Mulhearn 1937). The first systematic study of the disease was that of

Mackerras et al. (1940) in Australia. These authors established the parameters of the experimental disease using subinoculation of tissues and blood into susceptible cattle as the assay system. The next major advances were the isolation and characterisation of the causative agent as a rhabdovirus (van der Westhuizen et al. 1967). The first direct evidence that BEF virus multiplied in insects was supplied by Davies and Walker (1974) when they isolated BEF virus from a mixed pool of *Culicoides* species associated with cattle in Kenya.

The Disease and Apparent Anomalies

Ephemeral fever can vary in its clinical expression in individual animals. In mild cases the clinical signs may be limited to fever, loss of appetite, ocular and nasal discharge, muscle fasciculation and temporary lameness. In moderately severe cases there is depression, anorexia, loss of rumen motility, constipation, patchy subcutaneous oedema, joint swelling and a period of recumbency. In very severe cases, the animal may have paralysis of the limbs, resulting in recumbency. Salivation may be profuse. This phase

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may progress to loss of reflexes, coma and death or resolve suddenly. Uncommonly, there may be air under the skin of the backline. The sudden recovery from paralysis to normal mobility and absence of disabling pathology is incompatible with major direct cellular destruction by BEF virus. Variation in clinical signs is also seen in experimental cases even when animals are injected at the same time with aliquots of the same inoculum. This paper presents the results of recent research which provides a hypothesis of the natural history of ephemeral fever.

Fate of BEF virus after infection

For convenience, the discussion of the biological cycle is commenced at the point when BEF virus leaves the insect vector's mouthparts in saliva. *Culicoides* obtain their blood meal by lacerating the dermis and capillaries with their mouthparts. The insect's saliva, which may contain virus, mixes with the blood pool, which is drained from the site via the lymphatic system. This is likely to be an inefficient route of infection as experimental evidence suggests that the virus does not appear in lymph during disease and, except for one case reported by Mackerras et al. (1940), it is not possible to infect cattle by intradermal, subcutaneous or intramuscular injection of BEF virus. Although it is possible that local multiplication of BEF virus occurs in non-vascular intradermal tissue, this is likely to be self limiting because BEF virus is a potent inducer of interferon in bovine cells. This may explain the observation that BEF virus does not appear to multiply in cell cultures of bovine origin. In contrast to midges, mosquitoes probe the dermis to locate and penetrate small blood vessels before feeding. This parallels the experimental situation where intravenous inoculation of cattle is necessary to reliably reproduce disease. It is therefore likely that mosquitoes are more efficient vectors of BEF virus than are *Culicoides*.

After infection, the length of the initial cycle of multiplication of BEF virus is not well defined. BEF virus has been detected as early as 43 hours after inoculation using *Aedes albopictus* cell cultures as the assay system (Uren et al. 1992) and at 29 hours by subinoculation into cattle (Mackerras et al. 1940). The highest titre of virus in blood occurs approximately 24 hours before increases in neutrophil numbers or serum interferon levels and 36 hours before pyrexia and the earliest clinical signs (Uren et al. 1992).

The fraction of the blood in which BEF virus can be found in various stages of the viraemia, and the source of the virus are uncertain. Mackerras et al. (1940) found BEF virus in the leucocyte but not in the erythrocyte fraction. Young and Spradbrow

(1980, 1984) found viral antigen in neutrophils. Virus has also been detected in plasma (S.S. Davis, pers. comm.).

Mackerras et al. (1940) described one case where clinical disease began at 29 hours which would mean that virus rose to a titre sufficient to induce disease in less than 24 hours. A prepatent period of 29 hours, or less, is consistent with the virus replicating through 4 or 5 cycles entirely within the vascular endothelium. The vascular endothelium is completely exposed to the high levels of interferon which circulate during fever and which would inhibit BEF virus entering uninfected cells. In the period of 3–4 days between clinical recovery and antibody circulating in amounts which are adequate to neutralise free virus, the protection afforded by interferon would suppress or slow BEF viral replication. BEF virus contained on or within neutrophils in the bloodstream or serous cavities is probably there in the course of normal phagocytic function of those cells and is carried passively (Young and Spradbrow 1984).

The clinical signs and their cause

The clinical signs of ephemeral fever can be completely prevented without preventing viraemia by treatment with anti-inflammatory drugs (Uren et al. 1989). The clinical signs can be ameliorated by treatment in their earlier stages by anti-inflammatory drugs (Uren et al. 1989) or with calcium borogluconate or both (St George et al. 1984). As these drugs are not antiviral this clearly demonstrates that the host reaction is being modified.

The clinical signs and pathology can be considered in five groups. The first group are the toxic signs: depression and cessation of milk secretion. The second group are the inflammatory signs: fever, depression, anorexia, tachycardia, permeability of blood vessels, joint swelling, subcutaneous oedema, ocular and nasal discharges and neutrophilia. The third group are those due to hypocalcaemia: namely muscular tremor, ruminal atony, bloat, bowel stasis, constipation, incoordination, temporary paralysis of the limbs, loss of swallowing reflex (excess salivation and aversion to water are consequences), loss of palpebral and other reflexes and coma. The fourth is the subcutaneous emphysema in a small percentage of cases usually in hot weather (Theodoridis and Coetzer 1979). Emphysema is probably a consequence of the mechanical breakdown of lung tissue which in turn is a product of lung oedema, obstruction of alveoli and bronchioles after 2–3 days of overbreathing. Air migrates dorsally through septal tissue. The fifth sign is the prolonged paralysis which remains after fever terminates. The cow recovers in most respects, is bright and eating normally, but cannot stand for a period of weeks, or months. Recovery

may occur. This paralysis resembles the Guillain-Barre syndrome of humans, which follows a variety of viral infections.

The first three groupings of clinical signs and pathology will be considered in more detail.

Toxic signs

Animals with moderate to severe ephemeral fever appears dull and unresponsive. There is a sharp fall in milk production (Davis et al. 1984). The dullness may be a direct or mediated effect of interferon in the early stages of disease. This toxaemia later may be compounded or replaced by high levels of circulating ammonia. Toxic shock syndrome of women contains the same elements of direct systemic effect, caused however by a bacterial toxin, and induction of a hypocalcaemia (Wagner et al. 1981; Wick et al. 1982).

Inflammation

The presence of inflammation is evident in the gross and microscopic pathology (Basson et al. 1970) and the haematology and biochemistry (St George et al. 1984, Uren and Murphy 1985, Uren et al. 1992). The same biochemical changes occur in milk fever of cattle, where a reversible paralysis is also a feature. The high levels of interferon that occur in ephemeral fever could induce the inflammation through an interleukin cascade (Uren et al. 1987). As shown in Table 1, interferon induces most of the same effects as in experimental interferon toxicity in humans (Scott et al. 1981). Both conditions are treatable with anti-inflammatory drugs.

The primary effect of inflammation is an increased permeability of small blood vessels which has been

Table 1. A comparison of the toxic effects of synthetic interferon on human volunteers (Scott et al. 1981) with the signs observed in cattle with ephemeral fever.

Interferon toxicity in man	BEF virus infection cattle
Fever	Fever
Headache	?
Tachycardia	Tachycardia
Muscle stiffness	Possible muscle stiffness
Aching joints	Joint swelling
Malaise	Depression
Transient lymphopaenia	Lymphopaenia
Neutrophilia	Neutrophilia
Reduced plasma Zn	Reduced plasma Zn
Prevented with indomethacin	Prevented with phenylbutazone and salicylates

demonstrated to occur in ephemeral fever (Young and Spradbrow 1990). This produces lung oedema, a neutrophilia, plasma and cellular movement into tissues, joints, peritoneal, pleural and pericardial cavities, subcutaneous oedema and direct effects on muscle fibres. Temporary effects on fertility of bulls may be a direct effect of the generalised inflammation rather than virus effects on semen. BEF virus has not been detected in semen in natural disease (W.A. Snowden, T.D. St George, pers. comm.) Reduced semen quality is preventable by anti-inflammatory treatment. Rles can be detected on the lungs on the second day of fever when the ammonia level may be compounding oedema. The accompanying high plasma pH may slow virus replication as BEF virus deteriorates more rapidly at above or below pH 7.2 (Heuschele 1970).

The plasma biochemistry is that of an inflammatory disease (van Miert 1985) namely falls in plasma calcium, zinc, iron and inorganic phosphate, with rises in plasma copper, fibrinogen and glucose (Murphy et al. 1989; Uren et al. 1992). In experiments in mice infected with influenza virus where an inflammatory condition was created, Hurd et al. (1991) showed that zinc and iron accumulated in the liver. A rise in serum copper was attributed to increased synthesis of ceruloplasmin. Interleukin-I was cited as the mediator.

Hypocalcaemia

A clinical hypocalcaemia is detectable in two ways; reduced plasma calcium (< 2.1 μmol/L) and the almost immediate specific response of the clinical signs to infused calcium borogluconate in the same sequence as described with milk fever (Blood and Henderson 1974).

The uptake of calcium from the diet is dependent on a fully functional rumen. The plasma calcium levels begin to fall for several hours before ruminal movements stop. Bloat may follow and plasma ammonia levels rise sharply as a consequence. In cows where the swallowing reflex is lost, saliva drips on the ground and does not return nitrogen to the rumen in its normal function. These cows usually cannot drink water. Ruminal stasis largely prevents calcium adsorption into the body and thus compounds the problem. The excretion of calcium into urine and faeces is not increased. This has been measured by G.M. Murphy, T.D. St George and M.F. Uren (pers. comm.) by monitoring the mineral balances in cattle with ephemeral fever in metabolism crates.

There is insufficient time during the course of the disease for mobilisation of any of the large store of calcium in the bones. Mobilisation requires a week to be effective (Kronfeld 1971). In lactating cows,

milk secretion virtually stops during ephemeral fever (Theodoridis et al. 1973; Davis et al. 1984). This is a fortunate effect as it limits calcium loss in lactating cows. Thus we have a situation where calcium input and output from the body is reduced, there is displacement of unbound calcium to the protein bound fraction driven passively by high pH; there remains an overall temporary deficit in total plasma calcium. The high pH (which may be up to 7.8) is induced initially by loss of carbon dioxide from rapid respiration in the first febrile stage and later by high ammonia levels in the blood. Some calcium is taken up by the increase in the non-esterified fatty acid in plasma (Murphy et al. 1989). It is possible that the activated neutrophils account for the loss of the remainder. When neutrophils are activated calcium is displaced from their surface to the interior (Forehand et al. 1989) and is presumably replaced from the surrounding plasma. In severe cases of ephemeral fever, the excess fluid in joint capsules, peritoneal and pleural cavities is rich in neutrophils (Young and Spradbrow 1984), so that a large proportion of the activated neutrophils have left the circulation. Neutrophils do not return to the vascular circulation (Schalm et al. 1975). The mean time of arrival of granulocytes into the circulation is approximately seven days whether normal, or in calves given an endotoxin, and their half life in blood may be as short as 5 hours (Valli et al. 1971). The activated neutrophil population may thus function as a short term sink for calcium and would return their calcium and other minerals to the plasma as they finish their lifespan. The other temporary calcium sink could be the liver. Hurd et al. (1991) showed a rise of 33% (p < 0.001) in liver calcium levels in an experiment inducing inflammation with an influenza virus in mice.

The other disease of cattle where hypocalcaemia induces paralysis is milk fever, which also has an inflammatory base. It occurs in multiparous cows, usually within 1–2 days of parturition. The same inflammatory markers are present. These include neutrophilia, eosinopaenia, raised temperature (followed by a 1–2 °C drop), hyperglycaemia, increased plasma copper ammonia, and fibrinogen, decreased zinc, iron and phosphate levels (T.D. St George, G.M. Murphy, B. Burran, M.F. Uren, pers. comm.). In contrast to ephemeral fever, milk secretion continues in milk fever, thus producing a high mortality in untreated cows. The triggering mechanism of the inflammation in milk fever is presently unknown. On recovery from ephemeral fever there is some overshoot and plasma calcium levels rise above normal for some hours before homeostatic mechanisms normalise them.

Immunity

The general opinion is that one episode of ephemeral fever disease confers immunity on recovery. This immunity is sterile (Uren et al. 1992). However, there are ample field reports that second bouts of disease do occur in a small percentage of cases. There are three possibilities. The first is misdiagnosis of another viral disease. The second is recrudescence. This occasionally occurs in experimental disease where clinical signs recur a few days after recovery from the fever. The effect of interferon on cells is temporary (Stewart 1981) and its effect is measured in days. If the neutralising antibody response is slightly delayed, BEF virus could enter susceptible cells again and recrudescence could follow. The third possibility is reinfection of cows where only a limited primary antibody response occurred. This is consistent with an interval of some weeks between attacks. Accumulated experience is that cattle with even low levels of homotypic antibodies to BEF, as detected by the ELISA test (Zakrzewski et al. 1992), are immune to natural or experimental disease.

A constant feature of field reports is the observation that the second episode of ephemeral fever is always more severe than the first. Possibly the presence in the bloodstream of trace levels of antibody, which while inadequate to protect, enhance the ability of BEF or a related virus to enter cells. Such an effect has been shown with flaviviruses, and has been suspected with rabies (Celis et al. 1985).

Once protective levels of neutralising antibodies are established in the bloodstream, any further BEF virus inoculated by an insect vector will be neutralised. The question remains as to whether BEF virus can re-enter the bloodstream from some other tissue within the animal months later and create a new focus for insect spread? If this occurs, then an overwintering mechanism could be provided by a delayed recrudescence of virus in cattle. There is an argument against this occurring. In two experimental cases, BEF virus was not found in serial samples of lymphocytes or lymph during viraemia and early clinical disease (M.F. Uren, T.D. St George, and S.S. Davis, pers. comm.). This apparent absence of BEF virus from lymph is supported by the failure of Mackerras et al. (1940) to detect virus in mesenteric lymph nodes collected in the febrile period. Thus BEF virus, unless it infected an haemopoietic tissue, could not re-enter the bloodstream. If it were in haemopoietic tissue it would be exposed to circulating antibodies and be unlikely to persist. Mackerras et al. (1940) using susceptible cattle as his test system, found the blood of 10 cattle to be free of infective virus on the 4th and 5th days of con-

valescence, in contrast to the first three days. In a total of 24 cases, BEF virus has not been found beyond the 3rd day of convalescence, a maximum of seven days after experimental infection by insect tissue culture inoculation (M.F. Uren, T.D. St George and S.S. Davis, pers. comm.). If these two factors are taken into consideration then sterile immunity in cattle recovered from ephemeral fever is a reasonable assumption. The presence of BEF homotypic neutralising antibodies is thus contra-indicative of infection. Low levels of antibodies in cattle which will neutralise BEF virus *in vitro* are generated by Kimberley virus (Cybinski 1987). These are not protective *in vivo* (St George et al. 1984). The ELISA test distinguishes heterotypic from homotypic antibodies (Zakrzewski et al. 1992).

Vectors

BEF virus has been isolated from a pool of mixed species of African *Culicoides* (Davies and Walker 1974), *C. brevitarsis* in Australia (Cybinski and Muller 1990), a mixed pool of Culicine mosquitoes (St George et al. 1976), and twice from *Anopheles bancroftii* (Standfast et al. 1984; St George et al. 1976). The distribution of ephemeral fever in Africa, Asia and Australia far exceeds the combined distribution of all the species represented as sources of these isolates (St.George and Standfast 1989). There is some experimental evidence (H.A. Standfast, M.J. Muller pers. comm.) on multiplication in *Culex annulirostris*. BEF virus was detected between days five and eight post infection. This virus retains its virulence as demonstrated by one experiment using mosquito passaged virus to infect cattle. BEF virus has been shown by these workers to multiply in *C. brevitarsis* when experimentally infected. Isolation of BEF virus from an insect which has digested its blood meal is strong evidence that the virus has multiplied in that insect, but does not prove it can transmit virus to a cow. The necessity for BEF virus to enter the bloodstream directly, argues for mosquitoes and against *Culicoides* as efficient vectors.

There is epidemiological evidence from Australia that supports *Culex annulirostris* as a vector in addition to *An bancroftii* (Muller and Standfast 1986). Knott et al. (1983), in a three-year epidemiological study of ephemeral fever on one river system in northern Australia, found that transmission of ephemeral fever and seroconversion in sentinel cattle, occurred in the wet season (summer) when mosquito populations peaked and the opposite season to that favouring *C. brevitarsis* in the region. The close association of ephemeral fever epidemics

with recent rain or floods (Davies et al. 1975; Murray, 1970; St George et al. 1977; Uren et al. 1987) tends to indicate mosquitoes rather than *Culicoides* species as vectors in Australia as the response time of *Culicoides brevitarsis* populations is slower than mosquitoes > 3 weeks. The accumulating evidence points to multiple species of mosquitoes as the efficient vectors of ephemeral fever in Australia and *Culicoides* species as probably inefficient vectors.

In the epidemics of ephemeral fever of 1936–37, 1955–56 and three in the 1970s, the southward movement halted for the winter and resumed movement at approximately the same latitude in the following spring and summer (Seddon 1937; St George et al. 1977; Uren et al. 1983; St George 1985). The overwintering mechanism is not known. For the reasons argued earlier in this paper it is unlikely to involve cattle, as immunity is sterile. Unless an alternative vertebrate host is identified, mosquitoes remain the prime suspect. Adult females which have had one bloodmeal may live long enough to maintain the virus through the comparatively short winters of much of Australia. BEF virus grows more slowly in *Aedes albopictus* tissue cultures at lower temperatures (Hoffmann et al. 1985). Transovarial transmission is the alternative explanation. The link with recent rain would support this possibility.

Hypothesis of the Natural History of Ephemeral Fever

This section attempts to draw together the experimental observations into a unified hypothesis. The cycle begins when BEF virus is transmitted, principally by mosquito species, directly into the bloodstream of cattle. After being injected into a blood vessel the virus spreads throughout the vascular system within minutes and enters and multiplies in vascular endothelial cells. Newly replicated virus is immediately dispersed as it buds from vascular cells possibly endothelium, into the bloodstream in several cycles. High levels of interferon are generated in the process which produces two effects. The interferon renders both infected and uninfected vascular endothelial cells temporarily insusceptible to infection with BEF virus and slows replication, but the interferon also induces a toxaemia and a general inflammatory response in cattle with consequent clinical signs of fever, oedema, lameness and general malaise. The activated neutrophil response due to the inflammation allows many neutrophils to pass into the tissue spaces where they phagocytose BEF virus. Neutrophils do not re-enter the circulation and virus within them would

degenerate outside the bloodstream. The higher plasma pH which is the result of loss of carbon dioxide from overbreathing and high ammonia levels could reduce virus survival time as well as contributing to lung oedema.

Vascular endothelium exposed to interferon would be poorly responsive to residual circulating BEF virus for the 3–4 days after initial clinical recovery required for neutralising antibody levels to rise to an adequate level to combine with and neutralise the residual BEF virus. In a small percentage of cattle in which there is too long an interval between the decline of interferon levels and the arrival of neutralising antibodies, the cycle of cell infection, interferon production and clinical signs could recur. The eventual immunity is sterile and the blood is free of virus by 10–14 days.

The onset of hypocalcaemic signs and temporary paralysis is due to lowered plasma unbound calcium. The low ionised calcium level is induced by high blood pH high nonesterified fatty acid levels and an activated neutrophil shunt which could absorb calcium from the plasma and move it into tissues. The rapid recovery, so characteristic of ephemeral fever, follows the drop in viraemia and plasma interferon levels, the triggers for the inflammatory response and the consequential biochemical effects. Once the toxic stimulus of interferon diminishes recovery commences, BEF virus replication continues to decline due to impaired ability to enter and replicate in interferon affected target cells. The key biochemical change is a return toward normal of plasma calcium which effects both striated and smooth muscle control. The return of smooth muscle activity restores gastrointestinal function and all reflexes. As rumen regurgitation and swallowing normalise, the dependent biochemistry normalises. The temporary return to normal becomes permanent when neutralising antibodies enter the bloodstream in effective amounts. Once BEF virus is eliminated from the blood, immunity is sterile and in most instances lifelong.

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Epidemiology and Control of Bovine Ephemeral Fever in China

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Abstract

Epidemics of ephemeral fever have been recorded in China from as early as 1934. The disease was recorded in one of more provinces in 34 of the 43 years from 1949-1991. In 1991, an outbreak was confirmed by BEF virus isolation in Jilin Province, 44° N latitude, the most northern record of ephemeral fever in the northern hemisphere. In most epidemics the morbidity varied from 11-34% and mortality from 1-3%. The disease occurred in late summer and autumn and caused severe economic loss. Cattle were more severely affected than were buffalo. Research on effective vaccines began in 1976 after the first isolation of BEF virus in China. A vaccine based on mouse brain grown virus was abandoned because of side effects. Inactivated cell culture grown whole virus vaccine achieved protection rates of 50-80%. A detergent disrupted virus vaccine has given 93% protection in field trials in Guangdong and Shandong Provinces. Treatment of clinical cases combined western and traditional Chinese medicine and was based on antipyretic drugs, fluid and calcium replacement and nursing care.

BOVINE ephemeral fever is a viral disease of cattle and buffalo which occurs in Asia, Africa and Australia but not in the American continents.

Epidemics of ephemeral fever have been recorded in China since 1934 when the disease was known as bovine influenza. BEF virus was first isolated in China in 1976 by inoculation of mice and BHK21 cells with blood samples from affected cattle. Later, other isolates were obtained from cattle in Guangdong and Anhui provinces. This paper briefly reviews the epidemiology and control of ephemeral fever in China and expands the preliminary report of Bai et al. (1991).

Epidemiology

Epidemics of ephemeral fever

The earliest reports of ephemeral fever in China are of three outbreaks which occurred in Jiangsu province from 1934 to 1944. Epidemics were also recorded in the Wenzhou region of Zhejiang province in 1940, and in the Anhui region of Henan

province and around suburban Shanghai city from 1944 to 1949. It is now recognised that the disease in present in most parts of the country (Table 1).

When the disease occurs, large numbers of animals are affected and economic losses can be substantial. For example, in the four epidemics which occurred from 1954 to 1983, nearly 13.8 million cattle were affected (morbidity rate of 14%) and 100 429 deaths were recorded (case fatality rate of 0.7%). In some areas the morbidity rate was even higher, for example, in Ganyu county of Jiangsu Province, the morbidity rate was 40%.

In Guangdong Province, there have been 14 outbreaks from 1955 to 1991.

In the severe outbreak which occurred in Guangzhou city in 1983, 3124 of the 7831 cattle exposed were affected (morbidity 40%) and 141 (1.8%) affected cattle died. During 1991, on 18 commercial farms, 2572 (18%) of 14 666 cattle developed clinical signs and of these 121 (4.7%) died. In addition, 65 cattle aborted during the outbreak.

Epidemiological features of ephemeral fever

Provincial annual records show that whereas epidemics used to occur about every 10 years, inter-epidemic intervals of 3-5 or 6-8 years are now more

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Table 1. Years of recorded outbreaks in individual provinces and municipalities in China.

Province or city	Yearly occurrence
Guandong	1955, 1962, 1966, 1971, 1972, 1976, 1977, 1978, 1979, 1983, 1985, 1987, 1988, 1991.
Guangxi	1976, 1982.
Hunan	1955, 1963, 1966, 1978-1979, 1983-1984, 1987, 1988, 1989, 1991.
Hubei	1959, 1964, 1970, 1976, 1983, 1987, 1991.
Hainan	1957-1959, 1967-1969, 1971-1972, 1975-1984, 1985-1989, 1991.
Henan	1949-1989, 1991.
Jiangsu	1954-1955, 1966, 1976-1977, 1991.
Zhejiang	1955, 1958, 1965, 1971, 1983, 1987, 1988, 1991.
Fujiang	1954, 1955, 1958, 1963, 1966, 1972, 1975.
Jiangxi	1949-1989, 1991.
Anhui	1954, 1958, 1966, 1970, 1976, 1983, 1987, 1988, 1991.
Shandong	1954, 1955, 1959, 1965, 1966, 1970, 1971, 1976, 1983, 1987, 1991.
Shanghai	1952, 1955, 1958, 1971, 1976, 1983, 1991.
Yunnan	1965, 1973, 1975, 1977, 1978, 1982, 1983, 1986-1989, 1991.
Guizhou	1955, 1957, 1969, 1976, 1983.
Sichuan	1954, 1957, 1962, 1969, 1976, 1980, 1983, 1987, 1989
Tibet	1976-1979, 1985-1989.
Shausi	1953-1960, 1961, 1962, 1965, 1966, 1968, 1969, 1975-1978, 1982-1986, 1989, 1991.
Gansu	1956, 1964, 1969, 1975, 1977, 1981, 1986, 1987, 1989.
Ningxia	1957-1989.
Shensi	1954, 1959, 1991.
Beijing	1956, 1966, 1976.
Inner Mongolia	1966, 1971, 1974, 1975, 1976.
Liaoning	1983, 1991.
Jilin	1991.

common. In Guangdong Province the disease occurs even more frequently at 1–2 year intervals. Epidemics usually occur in late summer to early autumn. However, because China is a large country which spans several climatic zones, epidemics may occur at different times in different areas. For example, in 1991, an outbreak in Guangdong Province began in May (early summer) and the 1983 and 1985 outbreaks lasted for four months from June to November. In contrast, in Anhui Province in central China, epidemics usually begin in mid-year (July to October in 1983, July to November in 1987, June to October in 1988 and June to October in 1991). In Jilin Province in northern China the 1991 outbreak began even later in the year in August and lasted for only two months.

Rapid spread is another feature of ephemeral fever epidemics with geographical features such as mountains and rivers, providing no effective barrier. However, discontinuity has been reported and is difficult to explain. There is strong epidemiological evidence that BEF virus is insect transmitted. The virus has been isolated from *Culicoides* spp. in Africa and from *Culicoides brevitarsis* in Australia and Kenya (St George 1990). No BEF virus isolates have yet been made from insects in China.

Many reports (Inaba 1973; Standfast et al. 1973; St George 1990) have indicated that in the northern hemisphere, epidemic spread of ephemeral fever is confined to western Asia and to the south of latitude 38 °N. In the southern hemisphere, epidemics are rarely recorded further south than 25 °S in western and central Australia or 36 °S in eastern Australia. This distribution pattern is compatible with insect vectors that have climatic constraints on their distribution. In contrast to these findings, ephemeral fever was recorded in August 1991 in Jilin Province as far north as 44 °N (see Zhang Yalun et al. these proceedings). This is the most northerly report of ephemeral fever anywhere in the northern hemisphere and was probably associated with climatic variations favouring the dispersal of vectors. In order to better understand the role of *Culicoides* in transmitting BEF virus, Zhou (these proceedings) has studied distribution, reproduction and survival over winter in Anhui province.

High morbidity and low mortality are important features of ephemeral fever. In China, morbidity rates between 11–34% have been observed with case fatality rates of 1–3%. It has also been observed that the severity of clinical signs may vary depending on breed, age, sex, pregnancy state and density of cattle population. In general, adults show more severe signs than calves. Dairy cattle and Chinese yellow cattle are more susceptible to ephemeral fever than buffalo. For example, during the 1991 outbreak in Fengxiang County, Shanghai Region many cattle showed clinical signs but no disease was reported in buffalo even though serological tests showed that 69% of buffalo were infected.

In China, serological surveys have demonstrated that infection of cattle with rhabdoviruses related to BEF virus occurs. In one study, 4 of 420 (1%) serum samples had antibody to Kimberley virus, while 116 of 192 (60%) samples were positive for antibody to Berrimah virus and 63 of 131 (48%) samples were positive for Adelaide River virus. Studies in Australia indicate that although these viruses do not cause any clinical disease, there is antigenic cross reaction with BEF virus, which may lead to confusion in the serological diagnosis of ephemeral fever.

Control of Ephemeral Fever

Diagnosis

Effective control measures are based on specific diagnosis of the disease. In China the following procedures are recommended to assist in the specific diagnosis of ephemeral fever. Good clinical observation of affected animals during epidemics is important as ephemeral fever is easier to diagnose when several animals in a group show clinical signs. Haematology will assist the diagnosis; neutrophil numbers increase dramatically and haemoglobin levels may be up to three times higher than normal. Isolation of BEF virus should be attempted by intracerebral inoculation of 1–3 day old mice with leucocytes collected from animals in the early part of the disease. Isolates should be identified with specific antiserum in two-way cross neutralisation tests using reference viruses and antisera. Serum samples for serological examination should be taken from the affected cattle during fever and again during the recovery stage. Complement fixation, immunofluoresence, ELISA and neutralisation tests can detect antibodies against BEF virus. These tests have been established at the Harbin Veterinary Research Institute. The neutralisation test is now the most commonly used test to confirm a diagnosis of ephemeral fever. A fourfold increase in serum neutralising titre between the acute and convalescent sera is usually a good indicator of recent infection.

Prevention and control measures

Prevention of ephemeral fever should follow general principles, as for other infectious disease. Epidemic prevention stations should be used to supervise sentinel herds so as to provide evidence of virus activity before epidemics begin. Once the epidemic occurs, effective measures should be taken immediately to prevent the spread of the disease. State preventive regulations require the isolation of infected cattle, disinfection of cattle sheds and prohibition of the movement of infected cattle. Additionally, insecticides can be used to control insect vectors and animal sheds can be provided with insect screening.

Vaccination is an excellent way to prevent infectious disease, especially with a good quality vaccine.

Research into ephemeral fever vaccines has been carried out since 1976 when BEF virus was first isolated in China (see Liu Shanggao, these proceedings). Good protection was achieved by inoculating cattle twice with an inactivated mouse brain vaccine, prepared from strain 771214 isolated from Huixin in Guangdong Province (see Zang Zhejl, these proceedings). However, side effects proved to be unacceptable and a better vaccine was developed. Ephemeral fever vaccines are now prepared from the Beijing strain of BEF virus which is separately inactivated by several reagents, such as gentian violet, B-propiolactone and aluminium hydroxide. In laboratory experiments, the protective index ranges from 50% to 80%. Recently, we have developed a detergent disrupted vaccine which provides up to 93% protection. The vaccine was shown to be safe and effective when it was used in field tests in Guangdong and Shandong provinces (see Bai et al. these proceedings).

Treatment

Treatment is aimed at alleviating the clinical signs. During fever, Pyramidon and Analgin (trade names) are given via intravenous injection with fluid infusions to keep the fever down and avoid dehydration. Anti-inflammatory agents, in combination with various antibiotics, are also given routinely to prevent secondary infection by bacteria. Calcium therapy is necessary for stiff and lame cattle. The combination of western and Chinese traditional medicine is though to be useful.

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Studies on Bovine Ephemeral Fever in China, 1976-1977

Liu Shanggao*

Abstract

An epidemic of bovine ephemeral fever (BEF) occurred in the southern suburbs of Beijing between August and October 1976. BEF virus was isolated by suckling mouse brain passage. The virus was then passaged in BHK21 cell cultures for use in virus neutralisation tests for confirmative retrospective serology on naturally infected cattle. The cell culture adapted virus was designated 76 AMH. It has since become known as the Beijing strain or Beijing 1 and is the type strain of BEF virus for China.

DURING August to early October 1976, there was an outbreak of an acute infectious disease with high fever in dairy cattle of state farms, and beef and draught cattle in the suburbs of Beijing. This disease was characterised by a high temperature, respiratory signs and a drop in milk production. Most animals recovered in a few days. Some animals showed loss of swallowing reflex and paralysis. This paper presents the results of investigations of the cause of the disease and proceedures for prevention.

Materials, Methods and Results

Initial outbreak

The epidemic started in Yizhuang farm, in the southern suburbs of Beijing on 4 August 1976 and then spread quickly to 129 communities of 14 districts (or counties). According to (incomplete) records, the number of affected animals reached 10 266, of which 5945 cows belonged to 51 state dairy farms. The morbidity and mortality of cows in the 51 state dairy farms were 39% and 4% respectively. The epidemic ceased by the middle of October.

The epidemic in Hongxinbei dairy farm

More detailed investigations were able to be carried out on this farm where the epidemic started on 17 August 1976 and had ceased by October 10. The farm had a total of 352 cattle of which 162 (46%) showed clinical signs of disease and 13 (8%) of these

animals died. Among 211 adult cows there were 101 sick and 6 deaths, 49 young cattle with 19 sick and 3 deaths, 67 younger cattle with 13 sick and one death, 35 calves with 29 sick and three deaths.

The major signs observed were inappetence, listlessness, dullness and reluctance to move. In most animals, the temperature rose to 41-42 °C for 2-3 days and the heart rate increased up to 100 per minute. Most cattle had a nasal discharge and faeces were hard and dry. Two or three days later the temperature returned to normal and clinical signs gradually disappeared. Production of milk was greatly affected during acute disease and usually took some time to recover. Affected animals also occasionally had paralysis of the swallowing reflex which appeared after the acute period and some of these cattle died of inhalation pneumonia. Only a few cattle exhibited signs of lameness.

Transmission studies and virus isolation

Equal volumes of blood from two naturally affected animals (Nos 4210 and 7110) were mixed and homogenised. The mixture was then centrifuged at 3000 rpm for 15 minutes, and 10 ml of supernatant was inoculated into a healthy ox (No. 2) by intravenous injection. Red blood cells from another affected animal (No. 7) were also treated as above and inoculated into a healthy ox (No. 1). Three days post-inoculation, ox No. 2 had an elevated temperature of 41 °C which persisted for 2.5 days. Clinical signs were similar to the cattle from which the samples had been taken. Ox No. 1 showed no signs after inoculation. Seventeen ml of blood from ox No. 2, stored on dry ice for 144 days, were

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23

injected into a healthy cow (No. 18). Ten to 20 ml of blood from Cow No. 18 (with high fever) was inoculated into a healthy cow (No. 17) and then 10–20 ml of blood from cow No. 17 was passaged into cow No. 45 and a healthy buffalo (No. 22). Two to four days after inoculation, all cattle developed similar signs as ox No. 2.

For the isolation of BEF virus, white blood cells from animal No. 7 were inoculated intracerebrally into eight suckling mice, two were killed six days later, and tissue samples from those brains used to inoculate another nine mice, two of which showed signs of paralysis at 10 and 12 days post-inoculation. The brains from these mice were passaged and, as the passage level increased, the morbidity rate rose and finally stabilised at 100%. This isolate was designated 07 BAM. Similar treatment of a sample from ox No. 2 produced the same result and the isolate from ox No. 2 was designated 7110 BAM.

An emulsion of 8th passage level suckling mouse brain virus (76AM8) was inoculated into BHK21 cells. With each successive passage of 76AM8 in cells, cytopathic effect appeared earlier and earlier (30–40 hours). Virus which had only been passaged five or six times in suckling mice could not be adapted to BHK21 cell cultures. This adapted virus strain was designated '76AMH'. Injection of this virus into suckling mice could induce paralysis. None of the isolates could be successfully propagated in Vero cell cultures.

For the cell culture neutralisation test, all cattle sera were tested at the same dilution. The virus used was 16–24th passage level of 76AMH virus in cell culture and used at 10-fold dilutions. Twenty-one sera were tested, 20 were positive with a neutralising index of 320–10 000 which indicated cattle having serum antibodies to BEF virus.

Discussion

Investigation of an acute infectious disease occurring in August–October 1976 in Beijing, with fever and respiratory symptoms indicated that the characteristics of the disease were similar to those of bovine ephemeral fever described by foreign researchers. An ephemeral fever-like disease also occurred in dairy cattle near Beijing in August–October 1966, but a final diagnosis of that outbreak cannot be confirmed.

The epidemic of the disease in Beijing was apparently seasonal. By early October the epidemic of the disease ceased in all farms in the municipality of Beijing. Most cattle made a good recovery. Clinical signs were very similar to ephemeral fever reported in Japan, South Africa, and Australia. There was a bovine bluetongue-like disease (Ibaraki disease) reported in Japan which has similar symptoms as BEF. It is unknown whether Ibaraki disease also occurs in China. The disease identified in Beijing as ephemeral fever was obviously different from that caused by bovine respiratory syncytial virus, infectious bovine rhinotracheitis virus and bovine rhinitis virus. The occurrence of ephemeral fever was confirmed by the isolation of BEF virus which has since become known as the Beijing strain, or Beijing 1, and is the type strain for China.

Isolation and Identification of Bovine Ephemeral Fever Virus in China

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Abstract

Blood collected from cattle with clinical signs of bovine ephemeral fever was inoculated directly onto BHK21 cell cultures after treatment. This resulted in an isolate which was identified as BEF virus by electron microscopy, nucleic acid typing, physicochemical analysis and neutralisation tests, together with epidemiology and clinical signs.

EPHEMERAL fever is an acute infectious disease of cattle caused by an RNA virus in the family rhabdoviridae. The viral particle is bullet-shaped or cone-shaped and 85 x 130–220 nm in size. Outbreaks of disease suspected of being ephemeral fever occurred in Shandong, Jiangsu, Shanxi, Anhui, Hebei, Henan and Shaanxi Provinces in 1991. The morbidity rate was high and many animals showed severe clinical signs.

Materials, Methods and Results

Blood samples for virus isolation were aseptically collected into Alsever's solution from the jugular vein of clinically affected dairy cattle and sent to the laboratory on ice as soon as possible. The blood was centrifuged at 1500 rpm for 15 minutes and the supernatant discarded. Hank's balanced salt solution, with 500 units/ml penicillin and 500 μg/ml streptomycin, was added to the cell layer. The mixture was centrifuged at 1500 rpm for 15 minutes and again the supernatant was discarded. The sedimented cells were treated twice more by this method. Sedimented blood cells were resuspended in Hank's solution containing 0.5% lactalbumin hydrolysate as a 50% suspension. After three freeze-thaw cycles, the disrupted cells were centrifuged at 4000 rpm for 15 minutes, and the supernatant was collected for inoculation onto BHK21 cell cultures.

Cytopathic effects were not observed until day five of the third passage when cell rounding, shrinkage and detachment were seen, affecting up to 50% of the cell sheet. From passages 4–28, cytopathic effect first appeared three days post inoculation and by day five involved over 80% of the cell sheet. The virus from BHK21 passage seven was adapted to Vero cell cultures.

For electron microscopy, 50 ml of cell culture fluid from the eighth BHK21 passage showing over 80% cytopathic effect was frozen-thawed three times and centrifuged at 7000 rpm for 15 minutes. The supernatant was collected and centrifuged at 40 000 rpm for 1 hour, the supernatant discarded and the pellet resuspended in a small volume of buffer for negative staining with phosphotungstic acid. Scanning electron microscopy revealed bullet-shaped viral particles of 85 x 150 nm in size with morphology typical of a rhabdovirus. Truncated cone-shaped particles were also seen.

For nucleic acid typing, an inhibitor of DNA synthesis, 5-iodo-2-deoxyuridine (IUdR) was prepared (50 μg/ml in maintenance medium). The virus isolate and Aujeszky's disease virus (DNA virus as control) were serially diluted in ten-fold steps. Each dilution was added to eight wells of BHK21 cell cultures, four wells containing IUdR, and the other four wells as controls. After five days incubation the viral titres, with and without IUdR were calculated. Treatment with IUdR completely inhibited the growth of the DNA virus, Aujeszkys disease virus, but there was no effect on the field isolate, suggesting that this virus is an RNA virus. Treatment of the isolate with 0.5% trypsin, 5% chloroform or 20% diethyl ether did result in a loss

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of infectivity, suggesting that the virus is enveloped. Treatment of the isolate with buffers at pH 2 and pH 12 also resulted in a loss of infectivity. However repeated freeze-thawing did not affect the viral titre.

To evaluate the effect of defective interfering particles on viral titre in BHK21 cell cultures, passage of the virus undiluted, or diluted 1:100 was compared. The results are shown in Table 1. There was no appreciable difference in the titres produced by the two methods.

Table 1. The effect of inoculum dilution on virus titre at different passage levels ($\log_{10}$ $TCID_{50}$).

Inoculum	Passage						
	4	8	12	16	20	24	28
Whole culture suspension	4.25	4.5	4.5	4.75	5.0	4.25	4.5
1:100 dilution	5.0	4.5	5.0	4.75	5.0	4.5	4.75

For the serum neutralisation test, Chinese BEF reference virus (Beijing 1), Australian BEF reference virus (BB7721), and reference positive and negative sera were obtained from the Harbin Veterinary Research Institute. Ibaraki reference virus and positive serum were kindly provided by the Bluetongue Research Group, Animal Quarantine Institute, Ministry of Agriculture. The neutralisation test was a cell culture microtitre plate method. The results are shown in Table 2.

Discussion

BEF virus is known to be comparatively difficult to isolate. The method described in this paper is simpler and quicker than the traditional method of mouse brain inoculation. In contrast to the experience of other workers, no evidence was found for interference in viral replication when the virus was passaged without dilution. Isolation and identification of the field isolate confirmed the clinical diagnosis of infection with BEF virus.

Table 2. Neutralisation tests using antisera to BEF virus and Ibaraki virus.

Virus	BEF virus positive serum			BEF virus negative serum			Ibaraki virus positive serum		
	1:2	1:4	1:8	1:2	1:4	1:8	1:2	1:4	1:8
Field isolate	−	−	−	+	+	+	+	+	+
Chinese reference BEF virus	−	−	+	+	+	+	+	+	+
Australian reference BEF virus	−	−	−	+	+	+	+	+	+
Ibaraki virus	+	+	+	+	+	+	−	−	−

+ Viral cytopathic effect
− Inhibition of viral cytopathic effect

A Survey of Bovine Ephemeral Fever in Yunnan Province, China

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Abstract

Ephemeral fever was first described in Yunnan Province in Chinese Yellow cattle and in buffalo in 1964. Since then the disease has occurred many times, the most widespread epidemic being in 1982, when 2.2% of 6290 affected cattle died. The disease is seasonal, occurring between June and September. Until 1987, diagnosis was based entirely on clinical signs but is now supported by indirect immunofluorescence and serum neutralisation tests. A recent serological survey has shown a wide variation in antibody prevalence from county to county (5-75%) and with species (buffalo 55%, Chinese Yellow cattle 65%, and dairy cattle 5%).

BEFORE 1987, the diagnosis of ephemeral fever and epidemiological studies were based on clinical signs. After 1987, diagnosis was supported by indirect immunofluorescence and serum neutralisation tests. A serological survey was carried out in Yunnan Province to include the counties where ephemeral fever was unconfirmed. Results from epidemiology and serological tests have verified the existence of infection in most regions of the province. This report presents the results of a survey of cattle for antibodies to bovine ephemeral fever (BEF) virus in Yunnan Province.

Results and Discussion

Occurrence of ephemeral fever in Yunnan Province

Ephemeral fever was first described among buffalo and Chinese Yellow cattle (a beef and draught breed) at several villages in Fuyuan county in 1964. The total number of infected cattle was 569 and the average morbidity was 85%, but no deaths were recorded. Morbidity ranged from 50-100%. Since then ephemeral fever has been reported in many other regions. Before 1989, ephemeral fever had occurred at 67 townships in 17 counties of 6 regions mainly during June to September each year. The total number of the infected cattle was estimated at 32 200, with 637 (2%) deaths recorded. Average morbidity

rate was 82%. The most severe outbreak was in 1982 when ephemeral fever spread through 13 townships over seven counties in three regions affecting 6290 cattle with 137 deaths (2.2%). In contrast, only 180 (76% morbidity) cattle in only one township were affected by ephemeral fever in 1980 with the death of 8 cattle (4.4% mortality).

Clinical signs and postmortem lesions

Clinical disease in cattle was characterised by a body temperature of 40°-41 °C in the acute phase of the disease. Other signs included variable skin temperature, shivering with reduced temperature at the base of the horns, ears and hoofs. The conjunctivae were hyperaemic and there was an ocular discharge. Respiration rate was rapid, up to 80 per minute. The outside of the nose was dry and there was serous nasal discharge. The heart rate was as much as 60-90 beats per minute. Anorexia developed at an early stage and rumination had often temporarily ceased. Some cattle had oedema of the leg joints and lameness was often evident in all limbs. Milk production was reduced by more than 20%. At necropsy, most gross lesions were observed in the respiratory system. There was hyperaemia in the mucous membranes of the respiratory tract as well as oedema and emphysema of the lungs.

Serological diagnosis

Five hundred and twenty-three samples of cattle sera originating from 14 counties in eight regions of

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Yunnan Province were examined by indirect immunofluorescence for antibody to BEF virus, using reagents and methods of the Harbin Veterinary Research Institute. The results were as follows; 164 of 252 (65%) samples from Yellow cattle were positive; 127 of 231 (55%) samples from buffalo were positive and 2 of 40 (5%) samples from dairy cattle were positive. In cattle, the prevalence rate varied among counties and ranged from 5–75%. Nineteen samples collected from Yongsheng and Lijiang counties were examined for the presence of serum neutralisation antibodies to BEF virus at the Harbin Veterinary Research Institute; 14 (74%) samples were positive.

Discussion

The evidence from this serological survey for antibody to BEF virus shows that infection is seasonal being more prevalent during the hot rainy season. These are months of high activity for possible insect vectors. For example, during June to September in Yongsheng, Eryuan, Tuoping, Fuyuan and Malipo counties where there was a high incidence of ephemeral fever, the daily average temperature ranges from 17–23 °C. The annual rainfall is 112–192 cm and the relative humidity varies from 73% to 86%.

The sporadic occurrence of disease is another feature of ephemeral fever, with outbreaks every 2–5 years. For instance, four outbreaks of ephemeral fever were recorded in the 12 year period, 1978 to 1989 in Eryuan while three outbreaks occurred in the six year period, 1984–1989.

The disease is characterised by rapid spread with a short period of illness and a high morbidity and low mortality. Typically the whole herd in a village could be affected within 1–2 days and recover in 3–4 days. Ephemeral fever often recurs in the same region. For example, there were four outbreaks among a few townships in Youxin, Jiangwei, Dengyue and Eryuan counties, three outbreaks among Babao and Liancheng townships in Guangnan county and three outbreaks in Malicheng town of Mali county.

Many breeds of cattle are susceptible to ephemeral fever. In Eryuan county, dairy cattle are mainly affected, especially the 2–5-year old group. In other counties, Yellow cattle and buffalo appear to be infected at the same time.

Investigation of the Vectors of Bovine Ephemeral Fever Virus in Australia

M.J. Muller* and H.A. Standfast*

Abstract

The vectors of bovine ephemeral fever virus have been difficult to identify due to the sporadic nature of the disease, its short clinical phase, lack of premonitory signs and difficulties with isolation and identification of the virus. The approach to vector identification outlined in this paper is to determine the species of biting insects feeding on cattle in areas where the disease regularly occurs. A combination of *in vivo* and *in vitro* techniques are then used to determine which of the suspected vectors are able to transmit the virus. In Australia, both biting midges and mosquitoes appear to be capable of acting as vectors. However, vectors may be either very abundant with apparently low efficiency, for example *Culicoides brevitarsis* or uncommon but very efficient, for example *Anopheles bancroftii*.

BOVINE ephemeral fever occurs in Africa, the Middle East, Asia and Australia. It is a disease of cattle which subclinically infects a range of other ruminants. During epidemics, mortality rates are low but morbidity rates can be very high. Ephemeral fever was first recognised in Australia in 1936 in an epidemic form, and it subsequently spread rapidly through eastern Australia. Since then other major epidemics have occurred in 1955–56, 1967–68, 1972–74 and 1974–75. The rapid movement of these epidemics from the north to the south and east of the continent reinforced the conclusions of early studies that the virus was transmitted by flying insects. In the 1967–68 outbreak, the disease traversed over 2000 km in 8 weeks (Murray 1970). Major epidemics are now uncommon and evidence from sentinel herd studies has indicated that ephemeral fever is endemic in northern and eastern Australia (St George et al. 1977; Uren et al. 1983). The disease can radiate from localised focal points, as in the Hunter River Valley in the east of New South Wales (see Kirkland, these proceedings).

Studies by Mackerras et al. (1940) following the 1936 epidemic ruled out muscoid flies, black flies and March flies as potential vectors, mainly on the basis of their distribution and abundance. Doubt was cast

on the possibility of mosquitoes acting as vectors because limited experiments were unsuccessful. It was suggested that ceratopogonid biting midges were the most likely candidates but at that time the life cycles of these insects were poorly known, and methods for their collection and handling were not yet developed. Mackerras et al. (1940) were also limited in their capacity to work with the virus because at that time the only way to demonstrate the presence of BEF virus was to inoculate susceptible cattle and produce disease.

In 1968 concerted efforts were made to isolate BEF virus from mosquitoes and biting midges collected in association with outbreaks of disease in cattle in the field. By this time, knowledge of the taxonomy of the Australian biting midges was considerably improved and techniques were devised for the capture and handling of these minute insects (Dyce et al. 1972). In addition, it was possible to isolate virus by the intracerebral inoculation of suckling mice.

A number of attempts to determine the vectors of ephemeral fever were made by collecting biting insects associated with cattle during disease outbreaks. However, these attempts were largely unsuccessful. By the time the insect collecting team was mobilised and collection routines were set up at outbreak sites, the bulk of the disease activity was over. However, one isolation of BEF virus was made from a pool of 10 relatively rare mosquitoes of four species, collected during an outbreak at Rockhampton on the central Queensland coast (St George et

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al. 1976). Because of the restricted abundance and distribution of the species in the pool they could not have been more than incidental vectors of BEF virus.

In a change of tactic, a site was chosen where serological studies indicated that there was a high probability that BEF virus would be active each year. The site chosen was the Coastal Plains Research Station, 80 km southeast of Darwin in the Northern Territory. At this site, a program of continuous insect and serum collection was run for 18 months covering two monsoonal wet seasons and the dry season in between. Over this period, approximately 170 000 biting midges and 60 000 mosquitoes were collected and processed in species pools for virus isolation, in both cell cultures and suckling mice. From all of these, one isolation of BEF virus was made from a pool of 77 *Anopheles bancroftii* mosquitoes (St George et al. 1976). At the time the insects were collected, there were no clinical cases of ephemeral fever but there were seroconversions in cattle at the collection site. *Anopheles bancroftii* can be very abundant in the wet season in northern Australia, but the distribution of the disease is far wider than that of this species. In the meantime BEF virus had been isolated from a mixed species pool of *Culicoides* biging midges in Kenya (Davies and Walker 1974).

The mouse inoculation technique used to isolate BEF virus was both laborious and inefficient. For example, both the Kenyan isolation and the first Australian isolation required four passages in mice, while the isolation from *Anopheles bancroftii* required two mouse brain passages. In an attempt to increase the isolation rate of BEF virus, a different approach was taken in 1984 when collections of biting midges and mosquitoes were made on a dairy farm at Peachester, 90 km north of Brisbane, during an outbreak of ephemeral fever. Insect material obtained in 1984 was passaged through live *Aedes aegypti* mosquitoes, by intrathoracic inoculation (Muller 1987) followed by a 10-day holding period at 20 °C before further passage in BHK21 cell cultures. The same field insect material was also processed in parallel by direct inoculation into BHK21 cultures and by one or two passages in *Aedes albopictus* (C6/36) cell cultures before BHK21 cultures. Approximately 9000 mosquitoes in 227 pools and 14 400 biting midges in 157 pools were processed in this manner. Two isolations of BEF virus were made, one from a pool of four *Anopheles bancroftii* which had been collected feeding on a febrile cow and held for 12 days at 20 °C, and one from a pool of *Culicoides brevitarsis* collected in a light trap (Cybinski and Muller 1990). Both isolations were made only from material processed through live mosquitoes.

While *C. brevitarsis* has a more extensive distribution than *Anopheles bancroftii*, neither of these species are found in some inland areas where ephemeral fever disease has occurred, such as south-western New South Wales and parts of Victoria. In the laboratory, mosquitoes have been tested for their ability to become infected with virus by using artificial feeding techniques. BEF virus was recovered from 3 of 23 *Culex annulirostris* mosquitoes 12 days after feeding on a blood-virus mixture (Muller and Standfast 1986). When *Culex annulirostris* were inoculated with BB7721, the Australian reference strain of BEF virus, up to 70% were found to be excreting virus by *in vitro* capillary tube feeding after seven days incubation at 26 °C (M.J. Muller, pers. comm.).

Although many thousands of *Culex annulirostris* collected in the field have been processed for virus isolation by the inefficient system of intracerebral inoculation of mice, and many other viruses have been detected (Doherty et al. 1972, 1973; Standfast et al. 1984), no BEF virus has been isolated from wild caught insects of this species. However, the recognised distribution of *Culex annulirostris* bears a remarkable resemblance to the greatest known extent of ephemeral fever in Australia.

Thus it appears that BEF virus has a number of vectors in Australia, and may in fact be spread by more than one insect species during an outbreak (Muller and Standfast 1986). However there are still no species which satisfy the criteria for classification as confirmed vectors.

It is interesting to speculate, based on what is now known of the way the virus circulates in the vertebrate host, that mosquitoes are more likely to be involved as vectors. Biting insects may be categorised as either vessel feeders or pool feeders, depending on how they obtain their blood meal from the host. Most mosquito feeding is of the vessel type, where the stylets of the mouthparts are inserted directly into a blood vessel, probably a venule, under the surface of the skin. On the other hand, biting midges fit into the pool feeding category, where the surface of the skin is lacerated by the insect's mouthparts, causing blood to accumulate in a pool at and just below the epidermis.

In the vertebrate host, BEF virus circulates in blood before and during illness. Virus was not found in the lymph, or in the lymphocytes in the lymph, at least in the first two days of viraemia (M.F. Uren, S.S. Davis and T.D. St George, pers. comm.). A feeding mosquito which is excreting BEF virus in its saliva will, in most cases, deposit that virus directly into the blood vessel, duplicating the only successful experimental transmission route. *Culicoides* deposit

virus extravascularly where it will more likely be 'scavenged' by macrophages, be degraded and collected into the lymphatic system. Thus, a vessel feeding insect, such as a mosquito, is more likely to be a vector because it delivers virus directly to the target tissue.

However, this does not rule out the possibility of a biting midge transmitting BEF virus. As mentioned above, BEF virus has been isolated in Australia from *Culicoides brevitarsis*, a species which will feed on cattle in extremely high numbers, as many as 5000 per animal per night (Standfast and Dyce 1968). The isolation of BEF virus in Kenya came from a mixed species pool containing 4000 midges. Given a high level of attack, even if infection and transmission rates are low, there is a chance for the insect to introduce virus which will find its way into a blood vessel.

Field collection of insects is not a very cost effective means of identifying viral vectors. For example, to obtain one isolate of BEF virus, over a period of 18 months almost 58 000 mosquitoes and 170 000 *Culicoides* were processed (Standfast et al. 1984). A better use of resources is to study the vector competence of blood-feeding insects in the laboratory. The first step is to determine the species of mosquitoes and biting midges which feed on cattle in the areas where the disease occurs. This can be done using a range of methods such as animal bait, light traps and vehicle-mounted traps around livestock, and even Magoon traps. These species can then be collected in the field, brought to the laboratory and exposed to the virus by feeding either on animals which have been experimentally infected, or by feeding through membranes or other artificial substrates. After an appropriate incubation period these insects can be assayed for infection.

Since infection of an insect with an arbovirus does not automatically lead to transmission, the capacity of the species to transmit virus should also be tested in the laboratory by inoculating insects to produce 100% infection, and then allowing them to feed on susceptible animals after an appropriate incubation period. Inoculated insects can also be used to determine transmission rates using *in vitro* excretion techniques, such as capillary tube feeding, to assay individual insects (Muller 1987). The development of more sensitive ELISA tests and techniques such as the polymerase chain reaction, will increase the sensitivity of such assays.

While investigations of vector competence in the laboratory are more likely to produce results than attempts to isolate virus from field collections, insects will still need to be collected in the field for such laboratory trials. The insects being assayed at the end of a collection, manipulation and incubation program represent only 2–3% of the initial number collected. Better survival and feeding rates will usually be achieved with insects which are collected as immature stages or larval breeding substrates from the field. Insects from colonies can also be used, vector competence results from colony insects may not be an accurate reflection of the field situation for the same species. Colonies are also time-consuming and expensive to maintain.

Mosquitoes infected with BEF virus become permanently paralysed when they are anaesthetised with carbon dioxide (Figure 1). This type of sensitivity has been described for other mosquito species and viruses by Rosen (1980) and Turell et al. (1982). The incubation period for development of this paralysis is temperature dependent. When *Aedes aegypti* are inoculated with BEF virus and held at 26 °C, exposure to carbon dioxide at six days post-infection produces 100% paralysis. On post-infection day 16 and incubation at 15 °C, only 50% were paralysed, although the remaining 50% appeared to be affected to some extent. At 20 °C the effect is intermediate, with 100% paralysis on post-infection day 14, and some affected but not paralysed mosquitoes from days 6 to 10. The syndrome is most clearly seen at 26 °C, where there are no equivocal results. There may also be variation between species, as inoculation of *Culex annulirostris* with the same virus produced 94% paralysis after seven days at 26 °C. This effect is useful for detecting infection in mosquitoes without the need for tissue culture-based analysis. *Culoides brevitarsis* midges which have been inoculated with BEF virus do not appear to suffer any ill effects from anaesthesia with carbon dioxide.
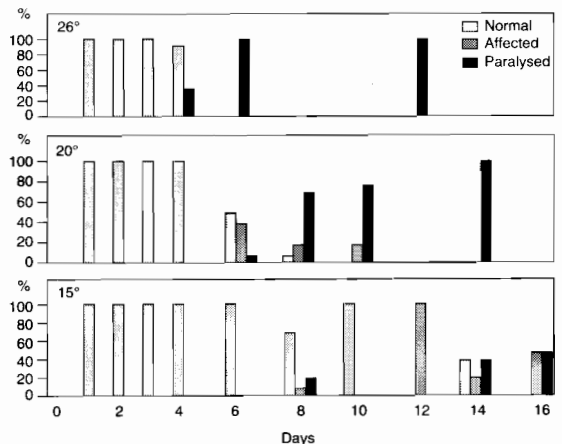

Fig. 1. The percentage of *Aedes aegypti* which are either normal, affected or paralysed following anaesthesia with carbon dioxide, on various days after infection with BEF virus.

It has been assumed that arboviruses do not have detrimental effects on their vectors. However it is now recognised that this is not always the case (Turell 1988). In preliminary trials it was found that infection of *Culex annulirostris* by inoculation with BEF virus (BB7721) led to increased mortality in comparison with controls inoculated with cell culture fluid (Figure 2).
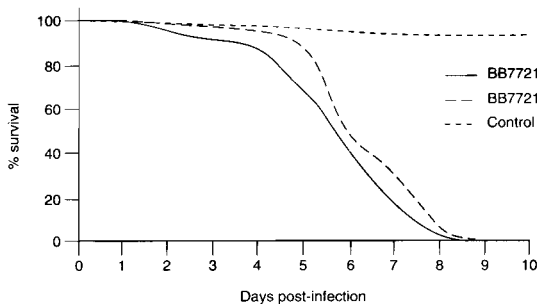
Fig. 2. The effect of inoculation with BEF virus on the survival of *Culex annulirostris*. Control mosquitoes were inoculated with cell culture fluid.

Further research needs to be carried out on the vectors of BEF virus. The definitive vector(s) have not yet been identified in any country where ephemeral fever occurs. Survival of the virus between seasons is not understood. There is no evidence of transovarial transmission of any rhabdoviruses apart from those spread by phlebotomine sandflies (Turell 1988). Survival in adult insects hibernating during the winter is a distinct possibility. The long-distance dispersal of infected insects on wind currents as a means of spread of the virus also needs further investigation.

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The Epidemiology of Bovine Ephemeral Fever in South-Eastern Australia: Evidence for a Mosquito Vector

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Abstract

Bovine ephemeral fever first appeared in Australia in 1936 as a major epidemic sweeping through the cattle population from the tropical north to the temperate southern regions. This epidemic pattern was repeated at regular intervals until 1975 with little evidence of disease in the intervening years. In central coastal New South Wales, this pattern changed to one of endemicity, alternating between years with significant disease outbreaks and a second or sometimes third year with sporadic cases. Even though the virus has become endemic in the central coast and Hunter Valley regions of New South Wales, with a somewhat regular pattern of transmission, there have been outbreaks of disease in areas quite remote from other cases. When the distribution of these epidemics is compared with the pattern of *Culicoides brevitarsis*-borne virus spread, there is little overlap, suggesting that bovine ephemeral fever virus, at least in these areas, was transmitted by a vector other than *C. brevitarsis*. The occurrence of disease under dry seasonal conditions is also consistent with a mosquito vector.

IN south-eastern Australia, in the States of New South Wales and Queensland, bovine ephemeral fever (BEF) is an important viral disease of cattle. Its economic effects are seen as a severe loss of milk production in dairy herds while in beef herds there can be poor growth and even death of young unweaned calves due to lack of milk from their dams. Occasionally deaths occur in either beef or dairy cattle, especially when heavy stock are infected. Significant interruption to breeding can occur when herd bulls are infected and experience a temporary infertility. This viral infection is also an indirect but significant cause of economic loss through disruption to international trade in live animals, semen and embryos.

Disease is usually observed during the mid-summer to late autumn, although sporadic cases have been observed at other times, including late winter–early spring. Major epidemics have usually occurred in association with periods of high rainfall. The association of infection with periods of warm weather and high rainfall has led to the hypothesis that the virus is spread by an insect vector. The pattern of BEF virus transmission in Australia has changed after a period of 40 years. Between 1936 and 1974, four major epidemics of disease, rapidly spreading from north to south, were recognised, with either few or no cases on the interepidemic years (St George et al. 1977). There was no evidence for the persistence of the virus in New South Wales or the other southern states of Victoria and South Australia during this period.

In 1974–75, a separate focus of infection was recognised in the Hunter Valley (New South Wales), independent of infection elsewhere in the temperate areas (St George et al. 1977; Kirkland, 1982). Since then BEF has become endemic with infection observed annually (with the exception of 1989–90) in coastal New South Wales, especially in the Hunter Valley and adjacent central coastal region (Uren et al. 1983, 1987). An epidemic year is succeeded by a period of low incidence and sporadic transmission. The epidemiology of BEF virus infection has been studied continuously from 1975 to 1992. This paper will review patterns of transmission over this period and compare them with those of viruses known to be transmitted by the biting midge, *Culicoides brevitarsis*.

Methods

Data on BEF virus transmission were obtained by monitoring of sentinel cattle and the investigation of outbreaks of disease.

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Sentinel cattle

Young cattle were selected and sampled according to established criteria (Kirkland et al. 1992) in regions throughout New South Wales to provide coverage of both the areas with potential for *C. brevitarsis* activity and the major inland river systems (Figure 1). Serum samples were tested by standard methods for antibodies to BEF virus (Cybinski and Zakrzewski 1983) and a range of arboviruses known to be transmitted by *C. brevitarsis* (Kirkland et al. 1992). Patterns of composite arbovirus infection were constructed as defined previously (Kirkland et al. 1992). Briefly, the proportion of animals which had been infected with one or more of the *Culicoides*-borne viruses was determined for each sentinel site. An animal was scored as positive whether it had been infected with only one or several viruses in the surveillance year. For these studies, each surveillance period covers one year, commencing in the spring of one calendar year and extending through to the winter of the following calendar year.
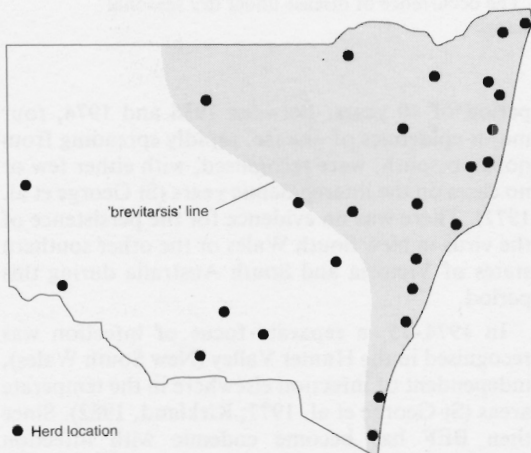
Fig. 1. The location of sentinel herds of cattle in New South Wales. The usual distribution of the midge *Culicoides brevitarsis* is depicted by the shaded area and its southern and western limits by the '*brevitarsis*' line.

Clinical investigations

Acute and convalescent serum samples were collected by veterinarians from animals suspected on clinical grounds to be infected with BEF virus. The acute phase sera were usually collected on day 1 or 2 of the illness and always within the first four days while the convalescent sera were collected between days 14–28. The paired sera were tested for specific anti-bodies to BEF virus using a microtitre virus neutralisation (VN) test (Cybinski and Zakrzewski 1983).

Interpretation of serology

A sick animal was considered to have been infected with BEF virus if the acute serum had a titre of < 8 and the convalescent serum had a titre of > 16 when the acute serum was negative or an eightfold or greater rise in titre when the acute serum had a low titre. The same interpretation was applied when determining whether an animal had seroconverted between successive samplings during routine surveillance of sentinels. Serological confirmation was sought for a proportion of sick animals each year.

Results

In most epidemic years BEF virus infection was confined to the coastal river plains and the associated valleys. A typical pattern of infection in an epidemic year is represented by that for 1985–86 (Figure 2) and for a year of sporadic occurrence by 1986–87 (Figure 3). However, in 1987–88, infection was detected in Northern Victoria (Shiel et al. 1989), approximately 500 km south of the nearest observed clinical cases and seroconversions in sentinel cattle (Figure 4). Atypical patterns of transmission were also detected in the 1990–91 and 1991–92 seasons. In 1990–91, an epidemic occurred both within the endemic area and on the central, south-western and north-western tablelands and slopes of New South Wales (Figure 5). In these areas, there were substantial deposits of surface water following extensive flooding during the winter and spring. Within the endemic area, some of the districts where there was an epidemic of BEF were almost drought stricken. In these areas, spread of the disease closely followed creeks and rivers. The pattern in 1991–92 was similar except that infections beyond the endemic area were only observed on the north-western slopes, mainly in areas where there were few cases the previous year but adjacent to areas where transmission had ceased the previous year.

For comparison with the BEF virus transmission pattern, a typical pattern of infection with Akabane virus is shown in Figure 6. The pattern of composite *Culicoides*-borne arbovirus infections for the same year (Figure 7) shows that infection with these viruses (belonging to the Simbu, bluetongue, epizootic haemhorragic disease and Palyam serogroups) was restricted to the *Culicoides* 'endemic' area. Similar patterns of *Culicoides*-borne arbovirus transmission were found in 1991–92 but at lower incidence.
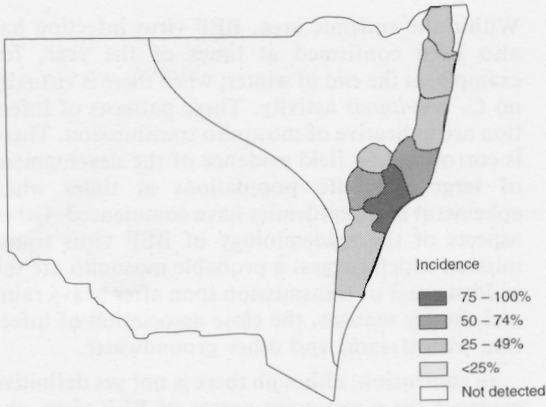
Fig. 2. The distribution of antibody to BEF virus in New South Wales in 1985–86.
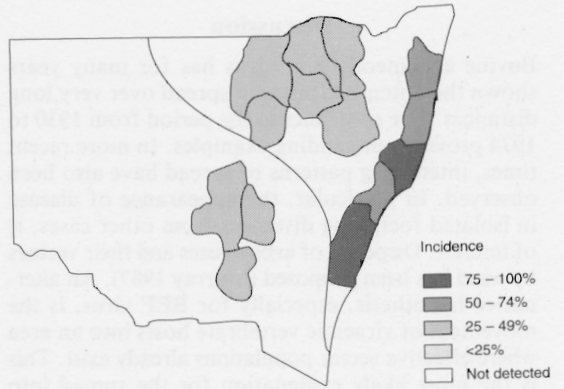
Fig. 5. The distribution of antibody to BEF virus in New South Wales in 1990–91.
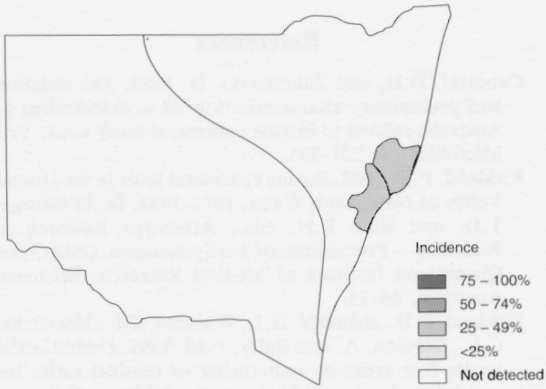
Fig. 3. The distribution of antibody to BEF virus in New South Wales in 1986–87.
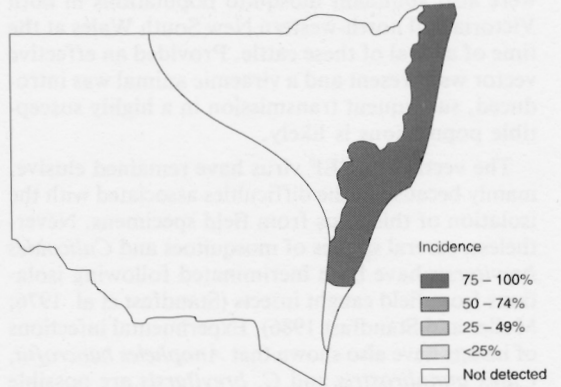
Fig. 6. The distribution of antibody to Akabane virus in New South Wales in 1989–90.
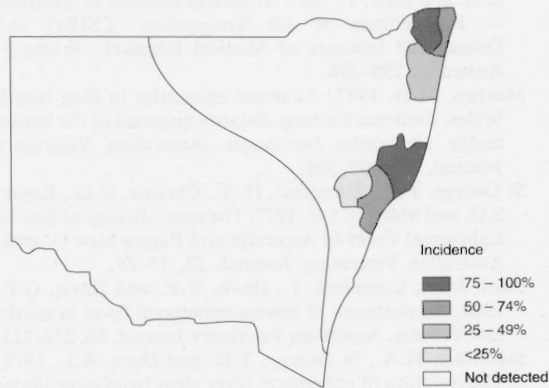
Fig. 4. The distribution of antibody to BEF virus in New South Wales in 1987–88.
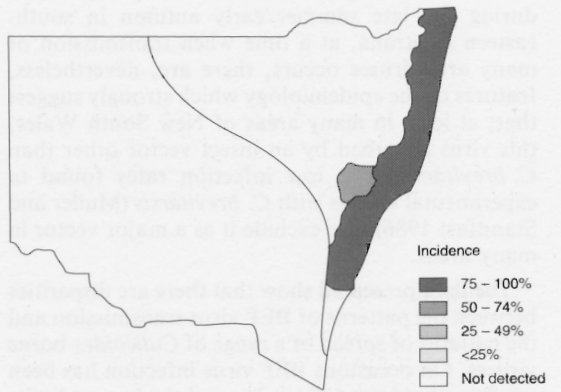
Fig. 7. The distribution of antibody to C. brevitarsis-borne arboviruses (Akabane, Palyam and EHD serogroups) in New South Wales in 1990–91.

35

Discussion

Bovine ephemeral fever virus has for many years shown the potential for rapid spread over very long distances. The epidemics in the period from 1930 to 1974 provide outstanding examples. In more recent times, interesting patterns of spread have also been observed. In particular, the appearance of disease in isolated foci, long distances from other cases, is of interest. Dispersal of arboviruses and their vectors by wind has been proposed (Murray 1987). An alternative hypothesis, especially for BEF virus, is the movement of viraemic vertebrate hosts into an area where effective vector populations already exist. This is the most likely explanation for the spread into northern Victoria in 1988 (Shiel et al. 1989) and into north-western New South Wales in 1991. In both cases, there are reports of the occurrence of disease soon after the arrival of cattle from the Hunter Valley and central coastal New South Wales. There were also abundant mosquito populations in both Victoria and north-western New South Wales at the time of arrival of these cattle. Provided an effective vector was present and a viraemic animal was introduced, subsequent transmission in a highly susceptible populations is likely.

The vectors of BEF virus have remained elusive, mainly because of the difficulties associated with the isolation of this virus from field specimens. Nevertheless, several species of mosquitoes and *Culicoides brevitarsis* have been incriminated following isolations from field caught insects (Standfast et al. 1976; Muller and Standfast 1986). Experimental infections of insects have also shown that *Anopheles bancroftii*, *Culex annulirostris* and *C. brevitarsis* are possible vectors (Muller and Standfast 1986) but the distribution of *Anopheles bancroftii* would suggest that it would only be a possible vector in Northern Australia. Although BEF virus is usually transmitted during the late summer/early autumn in southeastern Australia, at a time when transmission of many arboviruses occurs, there are, nevertheless, features of the epidemiology which strongly suggest that, at least in many areas of New South Wales, this virus is spread by an insect vector other than *C. brevitarsis*. The low infection rates found in experimental studies with *C. brevitarsis* (Muller and Standfast 1986) also exclude it as a major vector in many areas.

The data presented show that there are disparities between the patterns of BEF virus transmission and the patterns of spread of a range of *Culicoides*-borne viruses. On occasions BEF virus infection has been confirmed at least 500 km beyond the known limits of *Culicoides*-borne viruses in the same season.

Within the endemic area, BEF virus infection has also been confirmed at times of the year, for example, at the end of winter, when there is virtually no *C. brevitarsis* activity. These patterns of infection are indicative of mosquito transmission. There is corroborating field evidence of the development of large mosquito populations at times when ephemeral fever epidemics have commenced. Other aspects of the epidemiology of BEF virus transmission which suggest a probable mosquito are the sudden onset of transmission soon after heavy rains and, in dry seasons, the close association of infection with streams and other groundwater.

In conclusion, although there is not yet definitive evidence for a mosquito vector of BEF virus, the epidemiological evidence is strong. A likely candidate, with some experimental support (Muller and Standfast 1986), is *Culex annulirostris*.

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An Epidemiological Study of Bovine Ephemeral Fever in Ahui Province

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Abstract

Ephemeral fever is known to have occurred on at least ten occasions in Anhui Province, China, during the period 1947 to 1991. The disease usually occurs in the summer and autumn months, June to October. Until 1983, when BEF virus was isolated from cattle, diagnosis in cattle and buffalo was based on clinical signs. The animals which showed the most severe clinical response were dairy cows. In the 1983 epidemic the prevalence in dairy cattle was 50%, beef cattle 35% and buffalo 0.7%.

BOVINE ephemeral fever has been observed in Anhui Province on many occasions since 1947 but until 1983 the diagnosis was based on observations of clinical signs. In that year a virus was isolated in Hefei City from clinically affected cattle, and was identified as BEF virus by the Harbin Veterinary Research Institute. In 1987, a virus was isolated from cattle in Mengcheng County and was found to be a rhabdovirus by electromicroscopic examination. In 1991, virus was again isolated from cattle in Hefei City. This isolate was identified in a neutralisation test using antiserum to BEF virus provided by the Harbin Veterinary Research Institute. This paper describes the epidemiology of ephemeral fever in Anhui Province and includes information on clinical signs, disease dynamics and investigation of probable vectors.

Geographical Features of Anhui Province

Anhui Province of China is situated between 29°41'N – 34°38'N and 11°454'E – 11°937'E. It is an intermediate area between the Oriental Region and the Palaearctic Region according to zoogeographical classification. The Changjiang (Yangtze) River and the Huaihe River run from west to east, and divide the province into three natural zones; the Huaibei Zone (the zone to the north of the Huaihe River), the Jianghuai Zone (the zone between the Huaihe River and the Changjiang River), and the Jiangnan Zone (the zone to the south of the Changjiang River). The Huaibei Zone is a vast plain, while the Jianghuai Zone is hilly, and the Jiangnan Zone is mountainous, except for a narrow plain along the Changjiang River.

The average annual temperature in Anhui Province is 15°C, varying from minus 1°C in January to 28°C in July. In the Huaibei Zone, the average annual rainfall is 750mm, with 50–60% occuring in summer. In Jianghuai and Jiangnan Zones, the average annual rainfall is 1250 mm, with about 40–50% falling from May to July.

The Huaibei Zone is used for planting wheat and raising beef cattle while the Jianghuai Zone is used for planting rice and raising beef cattle and buffalo. To the south of the Jianghuai Zone only buffalo are raised. The Jiangnan Zone is used for planting rice and raising both beef cattle and buffalo, but there are only buffalo along the Changjiang River.

Disease Dynamics

Ephemeral fever has occcurred in Anhui Province at least 10 times in the last 45 years. The years when the disease is known to have occurred are: 1947, 1954, 1958, 1966, 1970, 1976, 1983, 1987, 1988, and 1991. The average interval between outbreaks is 4.9 years. In some years (1958, 1966, 1976, 1983, 1987 and 1991) the epidemics occurred in other provinces as well as Anhui Province. Ephemeral fever usually occurs from July to October (Table 1).

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Table 1. The seasonal occurrence of ephemeral fever in Anhui Province.

Year of occurrence	Date of initial case	Date of last case	Duration of occurrence
1976	4 July	28 Sep.	87 days
1983	20 July	15 Oct.	96 days
1987	5 July	16 Nov.	134 days
1988	25 June	21 Oct.	118 days
1991	20 June	27 Oct.	129 days

Table 3. Different age classes of cattle showing clinical signs in seven epidemics (percent).

Year of occurrence	Adults	Yearlings	Calves
1958	46.0	25.3	11.8
1966	26.1	12.5	36.4
1970	38.8	76.5	64.5
1976	35.9	72.1	36.5
1983	46.7	63.9	42.6
1987	4.1	3.7	2.7
1991	57.1	82.1	19.2

The general direction of spread of ephemeral fever in Anhui Province was from west to east. It was estimated that the speed of spread was 12 km daily in 1976, and 10 km daily in 1983. The rate of spread was found to be the same, even along the Changjiang River where the buffalo population was large. The disease occurred throughout Anhui Province, despite the uneven distribution of buffalo and cattle and the varying geography.

The exact source of infection for any of the outbreaks has not been determined. When the disease did enter a farm the number of cases increased rapidly to reach a peak. The spread of disease appeared to be faster in the 1976 outbreak than in the 1983 outbreak. Once the peak number of cases occurred, there was usually a gradual decline in the number of new cases but this period was usually longer than that leading up to the peak.

The morbidity varied depending on the kind of animal affected (Table 2). Buffalo were much less likely to develop clinical disease, but whether this is due to a different rate of infection compared to cattle, is not known.

Table 2. Different classes of animals showing clinical signs in four epidemics (percent).

Year of occurrence	Dairy cattle	Beef cattle	Buffalo
1983	49.6	35.3	0.7
1987	3.8	8.5	0.1
1988	59.5	20.8	0.8
1991	69.0	41.9	7.9

Another variable is age, with older animals being less likely to develop clinical disease (Table 3).

In an outbreak of ephemeral fever in Suixi County in 1983, the disease rates for different ages were: 29% for cattle less than one year old, 52% for cattle 1–2 years old, 62% for 3–5 year old animals, 32% for 6–7 years old, and 4.5% for animals greater than 8 years old.

Vector Studies

Eleven species of *Culicoides* have been collected in Anhui Province. They are: *C. antoni, C. arakawae, C. homotomus, C. maculatus, C. matsuzawai, C. mihensis, C. nipponensis, C. pulicaris, C. schultzei,* and *C. homotomus*. The dominant species were found to be *C. nipponensis* and *C. arakawae*.

Culicoides caught by net or light trap around stables or caught on the body surface of different animals were examined for blood-meal composition by immunoelectrophoresis. This demonstrated that *C. schultzei, C. homotomus* and *C. nipponensis* preferred the blood of domestic mammals (cattle, buffalo, asses, goats, sheep and swine), whereas *C. arakawae* preferred chickens.

The results of collection of *Culicoides* in the rice area of Ouzhong, Chuzhou ($32°15.4'$N, $118°18.3'$E) are provided for the following species: *C. homotomus,* first collected on March 19, final collection on October 13 (240 days), most prevalent between May 15 to July 13 (60 days); *C. nipponensis,* first collected on March 31, final collection on October 6 (190 days), most prevalent between July 31 to August 31 (32 days); *C. schultzei,* first collected on May 2, final collection on November 20 (203 days), most prevalent between July 13 to August 15 (34 days).

Blood-fed *Culicoides nipponensis* caught from the field were kept for egg-laying, hatching, larvae breeding, pupation and emergence under experimental conditions where 41–47 days was required for development from capture to emergence. The survival rate (number of adult insects emerged per number of eggs) was 4.7% with the main losses occurring during the larval stage.

To obtain more information about breeding sites and overwintering phases of *Culicoides*, 90 wet soil samples from rice and wheat fields were collected in the suburb of Heife ($31°14.3'$E). Larvae of *Culicoides*

were found in 50 samples. After emergence, they were identified as *C. homotomus, C. nipponensis* and *C. arakawae*, but no *C. schultzei* were found. Many active *Culicoides* larvae were collected in January from wet soil in rice fields, under a thin layer of ice when the air temperature was minus 5°C.

Discussion

Several outbreaks of ephemeral fever have occurred in Anhui Province, three of which have been confirmed by isolation of the aetiological agent. In the interval between outbreaks, ephemeral fever still occurred, but the cases were sporadic. When outbreaks did occur, they were often in summer and early autumn with rapid spread throughout the entire province. On the farms where infection is active, no infectious source could be identified, indicating that the disease is probably spread by insects. Dairy cattle and beef cattle are more susceptible to disease than are buffalo, but it is not known if this is a reflection of a difference in the infection rate. The dominant mammal feeding insect species in the region are *C. schultzei, C. homotomus* and *C. nipponensis* and these are the most likely vectors of BEF virus.

Bovine Ephemeral Fever in Indonesia

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Abstract

Ephemeral fever is considered an important disease of cattle in Indonesia. Serological surveys have shown that large ruminants throughout the country are infected. Monitoring of groups of sentinel cattle at intervals of 1000 or 2000 km across the country is starting to yield information on the seasonal pattern of infections, and also to allow opportunities for isolation of viruses.

INDONESIA, with its equatorial location, links the continents of Asia and Australia and has a variety of wet and drier tropical climates. The current paper reviews Indonesian reports of ephemeral fever and presents preliminary sero-epidemiological information of studies from BEF-group viruses.

Historical Perspective

The first report of ephemeral fever in Indonesia was by Merkens (1919) who described a new clinical syndrome in dairy cattle in Bandung, West Java which was consistent with the descriptions of three-day-sickness described in southern Africa and Egypt (Piot 1896, Bevan 1907). The next report of ephemeral fever in Indonesia was by Burggraaf (1932), who described cases in an epidemic between 1928 and 1931 on the east coast of Sumatra.

In 1978, an outbreak of ephemeral fever in East Java was investigated, (Soeharsono et al. 1982). Serum neutralisation tests showed that 22 of 25 animals had antibody to bovine ephemeral fever (BEF) virus. The disease persisted in the area for several years, and mortalities were at times quite high (Ronohardjo and Rastiko 1982). Clinical cases appeared to be still frequent in East Java in 1985 (Daniels et al. 1988). More recently another large outbreak of suspected clinical BEF has been reported, from the island of Kalimantan (Soleha et al. 1993a).

To promote the study of arboviruses in Indonesia, a sentinel herd program using groups of animals on several islands was commenced in 1987 (Sendow et al.1988, Daniels et al. 1991), and a serological capacity for the study of BEF-group viruses established (Soleha 1991; see Soleha et al. these proceedings).

Clinical Disease

The most complete clinical descriptions in Indonesia are from early reports. Merkens (1919) reported that dairy cattle said to be of Dutch and Australian origin were affected with a disease of sudden onset, high fever and increased respiration and heart rates. Inflamed conjunctiva, rumenal stasis, constipation, and muscular-skeletal lameness were also noted. Recumbent animals were observed and there were some mortalities.

Burggraaf (1932) reported a disease of sudden onset, with fever as high as 43 °C. There was inappetance, rumenal stasis and constipation, salivation, inflammation of the conjunctiva, lachrymation, increased heart rates and respiration. A shifting lameness caused by pain of joints and muscles was a characteristic of the disease. Cases becoming recumbent resembled parturient paresis. Aspiration pneumonia was a problem.

Epidemiology and Economics

Burggraaf (1932) reported a species difference in susceptibility with mortality being rare in *Bos indicus* cattle, and higher in dairy cattle. Of 80 dairy cows in one herd, 12 showed clinical signs, five died and

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four aborted. Of 27 heifers in the same herd, eight were affected and two died. No clinical signs were observed among 23 calves.

In East Java in the 1978–1982 outbreak, mortality was reported to be high, up to 36% of cases in the first year. Mortalities were fewer in subsequent years when a program of vaccination against haemorrhagic septicaemia and treatment of suspected cases with antibiotics was operational (Ronohardjo and Rastiko 1982). In the first year of the outbreak farmers were unfamiliar with ephemeral fever and may have slaughtered animals which they assumed would die.

In a rural economy based on smallholder farmers, such as in Indonesia, financial and economic costs of ephemeral fever are not only those of deaths and milk production losses. Cattle manure is an important source of fertilizer, and cattle are a major source of capital, personal wealth, fertiliser from manure and draught power (Ronohardjo and Rastiko 1982). The disease disrupts the farmer's efficiency and also his opportunity to earn extra cash through contract ploughing.

Burggraaf (1932) found the highest incidence of disease at the beginning of the wet season, when mosquitoes were abundant. Spread of disease occurred without direct contact, suggesting an insect vector. Ronohardjo and Rastiko (1982) noted that the spread of the disease in East Java was in the direction of the prevailing winds during the wet season, when monthly incidence of disease was highest. In the recent Kalimantan outbreak, peaks of disease were reported at the beginning and end of the wet season (Soleha et al. 1993a).

Current Studies

Procedures

A program for the study of arboviral infections of livestock in Indonesia (Daniels et al. 1991) includes ephemeral fever. The program is based on monitoring sentinel cattle, pigs and chickens and collecting samples for serology and virus isolation. Insects are also collected at sentinel sites, and identified to species, with the emphasis on *Culicoides* spp. Insects collected close to the laboratory are processed fresh for virus isolation, while those at distant sites are collected into alcohol.

Because of the problem of cross-reactions among BEF-group viruses in serum neutralisation tests (Cybinski 1987) a range of BEF group viruses for which reagents were available, were obtained from CSIRO Long Pocket Laboratories, Australia. These included BEF virus strain BB7721 (Doherty et al. 1969), Berrimah strain DPP63 (Gard et al. 1983),

Kimberley virus strain CS368 (Cybinski and Zakrzewski 1983) and Adelaide River strain DPP61 (Gard et al. 1984).

Serological surveys

Cattle sera obtained from a serum bank were tested for neutralising antibodies to BEF virus at a dilution of 1:4 according to the method of Soleha (1991). The results, presented in Table 1, show that infection with BEF virus occurs throughout Indonesia. The results of more intensive serological surveys undertaken in Irian Jaya and Timor (Soleha et al 1993b) are presented in Table 2. The cattle sampled in consecutive years were from the same herds. All districts studied were coastal, except for Jayawijaya, which is in the central highlands of Irian Jaya. The results confirm that BEF or closely related viruses, are widely spread in eastern Indonesia.

Table 1. Prevalence of serum neutralising antibodies to bovine ephemeral fever virus in cattle in several provinces of Indonesia.

Province	Cattle	
	No. tested	No. antibody positive (%)
Aceh (Sumatra)	55	11 (20)
Lampung (Sumatra)	55	18 (33)
West Java	40	11 (28)
Central Java	55	14 (26)
East Java	24	9 (38)
Bali	47	6 (13)
Nusa Tenggara Barat	55	15 (27)
Nusa Tenggara Timur	29	8 (28)
South Kalimantan	55	14 (26)
South Sulawesi	18	3 (17)
North Sulawesi	39	7 (18)
Irian Jaya	55	9 (16)
Total	**527**	**125 (23.7)**

Considering first the districts surveyed in Irian Jaya, Jayapura is a high rainfall area, 2750 mm per year, with rainfall usually not falling below 150 mm in any month. In this district the sero-prevalence in over 120 cattle in the two consecutive years was the same (24%). In the drier district of Merauke, on the south coast adjacent to northern Australia, the rainfall is 1750 mm per year and occurs predominantly in a four-month wet season, with an eight-month dry season. Here the sero-prevalence was similar each year (7–9%), but much lower than in wetter Jayapura. In contrast, the district of Kupang has a much lower rainfall (1250 mm per year), but the sero-prevalence (14%, 42%) in the two years of the study

Table 2. Prevalence of serum neutralising antibodies to bovine ephemeral fever virus in cattle in eastern Indonesia — including a comparison of data collected in two successive years.

Province/District	1989 % antibody positive (No. tested)	1990 % antibody positive (No. tested)
Irian Jaya		
Jayapura[1]	24% (122)	24% (156)
Jayawijaya	0% (9)	—
Merauke[1]	9% (86)	7% (128)
Fak Fak	2% (28)	—
Sorong	—	25% (28)
Biak Numfur	—	44% (25)
Nusa Tenggara Timor		
Kupang[1]	17% (105)	42% (113)

[1] Sentinel cattle sites

— Data not available

was higher than for Merauke, and showed considerable variation between years, perhaps indicating an effect of season. Merauke is more isolated than Kupang. It is separated from the Jayapura district by a wide mountainous area, Jayawijaya, where antibodies to BEF virus have not been detected (Table 2). The cattle population of Timor, where Kupang is placed, is approximately half a million, while the cattle population of Merauke is approximately 10 000. The cattle population of the south coast of Irian Jaya is being increased with imports from other provinces, especially Nusa Tenggara Timor (Kupang) and Nusa Tenggara Barat.

Seroconversions in sentinel cattle

To provide more information on the role of other viruses in the BEF virus group, detailed studies were carried out in Bali, Kupang and Jayapura, using Bali cattle (*Bos javanicus*). The sera were tested at a dilution of 1:4 in a serum neutralisation test (Soleha 1991).

The data available for Bali are for 1989–1990. In one animal, seroconversions were observed to Kimberley virus in March and to both Berimah virus and BEF virus in April. In December another animal seroconverted to BEF virus alone. In February individual calves seroconverted to either Kimberley virus or Adelaide River virus, and in March calves seroconverted to Kimberley virus and Adelaide River virus. It seems probable that several different BEF group viruses were circulating in the study area during the mid to late wet season. Not all animals seroconverted which may indicate inefficient vector transmission.

Seroconversions were recorded in two groups of calves at Kupang and Jayapura in 1990–1991. Patterns were similar to those in Bali. In Kupang one calf seroconverted to BEF virus, Berrimah virus and Kimberley virus in the same month, while another calf seroconverted to BEF virus and Berrimah virus at the same time. Several animals seroconverted to Berrimah virus at the end of the wet season, and one seroconverted to Adelaide River virus in July in the early dry season.

In Jayapura, with its much wetter climate, seroconversions were again in the period March to July, the end of the wet season. Most animals in the group seroconverted to Berrimah virus in June and July, with only four seroconverting to BEF virus. Also, during the observation period, most calves in the group seroconverted to Adelaide River virus, but over a longer period, January to September. In spite of the problem of virus cross-reactions complicating serological interpretations, at least two different patterns of seroconversions to different viruses were observed, which again suggests that more than one BEF group virus was circulating in the district, and also that the viruses may be preferentially spread by two different vectors.

In Bali and Kupang, separated from each other by over 1000 km, seroconversions to BEF virus and Berrimah virus occurred first in December, and then in March, April and May. In Jayapura the seroconversions to these antigens were again in December, then in April and May, reaching a peak in June and July.

Discussion

Serological surveys have demonstrated that infection with BEF virus is widespread in Indonesia. No clinical disease has been reported in the sentinel animals monitored to date. Although earlier reports were of disease in *Bos taurus* cattle, (Merkens 1919), with *Bos taurus* being more severely affected than *Bos indicus* (Burggraaf 1932), subsequent reports (Soeharsono et al. 1982, Ronohardjo et al. 1982) described clinical disease in local *Bos indicus*. Recent outbreaks of ephemeral fever-like disease in South Kalimantan involved many Bali cattle (*Bos javanicus*).

It is not clear whether BEF virus causes disease in water buffaloes (*Bubalus bubalis*) (Young 1979). None of the published reports from Indonesia mention this species, although a high prevalence of serological reactors has been found (see Soleha et al. these proceedings).

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A Study of Bovine Ephemeral Fever
Group Rhabdoviral Infections in West Java, Indonesia

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Abstract

The bovine ephemeral fever group of viruses includes, among others, bovine ephemeral fever, Berrimah, Kimberley and Adelaide River viruses. Serological studies of these four viruses were conducted in West Java to obtain preliminary information on the prevalence of infection in cattle and buffalo. Groups of sentinel cattle were monitored weekly to establish the seasonal incidence of infections. Viruses were isolated from sentinel cattle and from pools of *Culicoides* and mosquitoes. Serological evidence of infection with all four viruses has been found in cattle or buffalo. Some sheep and goat sera also contain antibodies to bovine ephemeral fever group viruses. Isolations of viruses included several that may be placed in the ephemeral fever group, subject to confirmation.

EPHEMERAL fever is caused by a rhabdovirus, bovine ephemeral fever virus, and has been reported from countries in Africa, Asia and Australia (St George, 1981). Clinical disease in cattle has been reported in Indonesia since early in the century, in 1918 and from 1928 to 1931 (Merkens 1919, Burggraaf 1932).

Although cattle are believed to be the primary host, antibodies to BEF virus have also been detected in some other ruminants; red deer (*Cervus elaphus*) and water or swamp buffalo (*Bubalus bubalis*) in Australia; waterbuck (*Kobus ellipsiprymnus*), wildebeest (*Connochaetes taurinus*), hartebeest (*Alchelaphus buselaphus*) and African buffalo (*Syncercus caffer*) in Africa and swamp buffalo in Malaysia (St George, 1988).

This paper reports further studies of BEF virus infections in West Java, the province where ephemeral fever was first reported in Indonesia (Merkens 1919). A serological survey of livestock in West Java for antibodies to several BEF group rhabdoviruses, BEF, Berrimah, Kimberley and Adelaide River viruses, was conducted and sentinel cattle were also monitored. Isolations of BEF group viruses were

successfully attempted from sentinel cattle, and some collections of insects at the sentinel sites were made.

Materials and Methods

Sera collected in West Java from 1986 to 1992, from various species of livestock including buffalo, sheep, goats, horses, chickens and ducks, were accessed from a serum bank. A small stratified serological survey of cattle was also conducted in several districts in West Java specifically for the present study.

Sentinel cattle have been monitored in West Java since 1987 at Depok, 95 m above sea level and with an annual rainfall of 3000 mm. Holstein-Friesian cattle were bled either weekly or monthly, commencing when cattle were approximately three months of age.

Insects for attempted virus isolation were collected weekly at the sentinel site starting in 1991. Light traps were run for three to four hours at dusk, collecting into a buffer solution with detergent which was then kept cool overnight. Insects were identified and separated into species pools for virus isolation.

The first technique of virus isolation attempted was that described by St George et al. (1978), as modified by Sendow et al. (1989). Uncoagulated heparinised blood was centrifuged at 1500 rpm for 10 minutes to separate the white blood cells (WBC) from other fractions of the blood. Cell culture monolayers of 4×10^5 BHK21 cells in 2 ml of

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minimum essential medium (MEM) (Flow Laboratories) containing 5% foetal bovine serum (FBS) (Flow Laboratories) and 200 units of kanamycin in roller tubes were inoculated with 0.1 ml of WBC from the buffy coat. The medium was changed with MEM containing 2% FBS and 200 units of Kanamycin 24 hours after inoculation. Cultures were incubated at 37 °C and observed daily. After 5 days, cultures were passaged to new BHK21 roller tubes, using 0.1 ml of cell suspension as the inoculum. After three passages, cultures showing cytopathic effect were supplemented with 10% FBS, and stored at − 70 °C and in liquid nitrogen. Cultures not showing cytopathic effect were discarded.

Another technique for virus isolation using chicken embryos (Gard et al. 1988) has been used since 1990. Approximately 0.025 ml of whole blood diluted in phosphate buffered saline (PBS) was inoculated intravenously into embryonated chicken eggs. The eggs were incubated at 33.5 °C and observed daily for 5 days. Dead embryos were harvested and the tissues homogenised and suspended in 2 ml of MEM with 5% FBS. Embryo suspensions were filtered through 450 μm (Millipore) and 0.1 ml of the filtered suspensions inoculated into monolayers of *Aedes albopictus* cells (C6/36) (St George 1985), and incubated at room temperature for seven days. Cell suspensions of infected C6/36 cells were passaged blind into monolayers of BHK21 cells in roller tubes.

Insect pools were processed in glass homogenisers in 2 ml of PBS at pH 7.2. Virus isolation in chicken embryos and C6/36 cells was used for virus isolation from insect suspensions.

Isolates were identified to group level by the indirect immunofluorescence (IIF) test using the method of Cybinski and Zakrzewski (1983). Infected cultures of BHK21 cells on spot slides showing cytopathic effect were fixed with 50% acetone for 20 minutes. Slides were air dried. Viral antigens were detected by adding a drop of anti-BEF virus (strain BB7721) mouse ascitic fluid (maf) and incubating for 30 minutes. Slides were washed three times with PBS and air dried. A drop of rabbit anti-mouse FITC at a dilution of 1:16 was added and incubated at 37 °C. After 30 minutes slides were washed three times with PBS, and once with distilled water. Stained cultures were read with a fluorescence microscope. Isolates reacting with antibodies to BEF virus in the immunofluorescence test were further tested in microneutralisation tests with polyclonal antibodies to BEF virus (Doherty et al. 1969), Berrimah virus strain DPP63 (Gard et al. 1983), Kimberley virus strain CS368 (Cybinski and Zakrzewski 1983), and Adelaide River virus strain DPP61 (Gard et al. 1984). Type viruses and antisera were supplied by CSIRO Long Pocket Laboratories, Australia.

Neutralisation tests were used for detecting antibodies to the BEF-group viruses in survey and sentinel sera collections, as listed above, and also for preliminary identification of isolates using methods previously described by Burgess (1974), Cybinski et al. (1978), and modified by Soleha (1991). Vero cells at a concentration of 2×10^5 cells per ml in MEM with 5% FBS were used. Viruses were propagated in monolayers of BHK21 cells and diluted to 100 $TCID_{50}$.

Results

Serology

Antibodies to BEF-group rhabdoviruses were detected in several species of livestock (Table 1). Reactors to BEF virus were found in cattle (15%) and buffalo (17%); to Berrimah virus in cattle (10%), buffalo (29%), sheep (13%), and goats (3%); to Kimberley virus in cattle (20%), buffalo (29%), goats (2%), and horses (8%); and to Adelaide River virus in buffalo (2%) and goats (23%). Data on the

Table 1. Detection of serum neutralising antibodies to bovine ephemeral fever (BEF), Berrimah, Kimberley and Adelaide river viruses in livestock in West Java.

Virus	Species						
	Cattle	Buffalo	Sheep	Goats	Horses	Ducks	Chickens
BEF	35/240	24/145	0/56	0/136	0/93	0/36	0/58
	(15)	(17)	(0)	(0)	(0)	(0)	(0)
Berrimah	16/157	12/42	3/24	3/105	0/83	0/36	0/58
	(10)	(29)	(13)	(3)	(0)	(0)	(0)
Kimberley	32/157	12/42	0/30	2/90	7/93	0/36	0/58
	(20)	(29)	(0)	(2)	(7.5)	(0)	(0)
Adelaide River	0/89	1/42	0/50	3/13	0/83	0/36	0/58
	(0)	(2.4)	(0)	(23)	(0)	(0)	(0)

Notes: Results are expressed as **No. reactors/No. tested** animals (figures in parenthesis are percentage of reactors)

distribution of infection at various altitudes showed a trend for higher prevalences at lower altitudes (Table 2). Because of the small number of sera processed and the small number of sites sampled, results were not analysed for statistical significance.

Sentinel cattle have been monitored since 1987 and tested monthly for antibodies to BEF-group viruses. Cattle were owned by various smallholder farmers, and difficulties in continuity of sampling were experienced. For example, in the period 1987–1988, the number of cattle monitored totalled 25, but only 10 cattle could be bled monthly for a full year. Antibodies to BEF virus have been detected in sentinel cattle each year. In 1987–1988 maternal antibodies were detected in 5 of 10 calves but were not detectable after cattle were 6 months of age. Seroconversions were seen in 8 of 10 cattle. In the period 1988–1989 one of 6 cattle had maternal antibodies, and 5 seroconverted. In 1989–1990, 5 of 11 cattle had maternal antibodies, and 6 seroconverted (Tables 3 and 4). The month in which seroconversions were

detected each year varied but was most frequently in the period December to July (Table 4), from soon after the start of the wet season through to the end of the wet season.

Insects for virus isolation were collected in Depok and Cisarua from 1991. The insects identified and the groups for virus isolation are presented in Table 5. *Culicoides* spp. were identified to species, but mosquitoes to genus only.

In 1987–1988 and 1988–1989 the isolation system was inoculation of samples into BHK21 cell cultures for three passages. In subsequent years the isolation system was inoculation of samples into embryonated eggs, followed by passage in *Aedes albopictus* cells and three times passage in BHK21 cell cultures in rotating tubes. The number of isolates from sentinel cattle blood samples varied each year. Not all such isolates would be expected to be BEF-group viruses. Nine isolates were obtained in 1988, two in 1989, thirty seven in 1990, and three in 1991 (Table 6). A total of 51 isolates were made from blood samples

Table 2. Detection of serum neutralising antibodies to BEF-group viruses in cattle at various altitudes in West Java.

Place	Altitude (metres)	No. of Cattle	BEF	BRM	KIM
Garut	<100	31	29%	16%	32%
	600	17	0	0	0
	1240	18	6%	0	33%
Tasikmalaya	<100	16	56%	31%	50%
	400–600	14	43%	21%	57%
Indramayu	<100	28	46%	29%	21%
Majalengka	400	20	25%	10%	10%

Table 3. Detection of neutralising antibodies to BEF virus in sentinel cattle at Depok, West Java in the period 1987–1988.

No. Cattle	1987							1988									
	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct
12	+	+	−	−	−	−	−	±	+	−	−	−	−	+	+	+	
13	−	−	−	−	−	−	−	+	±	±	+	−	−	+	+	+	
15	+	±	+	±	−	−	−	−	±	+	−	−	+	−	−	*	
16	−	−	−	−	−	−	−	−	−	−	−	−	−	*	*		
17	−	−	−	−	−	−	−	+	−	+	−	−	+	+	*	*	
19	+	+	+	+	+	+	+	±	+	+	*	+	+	−	−	*	
45	*	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+
46	*	−	−	−	−	−	−	−	−	−	−	±	+	−	+	*	*
47	*	+	−	−	−	−	±	−	+	+	+	−	+	+	−	+	+
49	*	*	*	+	+	−	+	−	−	−	−	−	±	+	−	*	*

+ Titre more than 4
± Titre less than 4
− Negative
* Not available

47

collected at the Depok sentinel site in West Java. Three isolates were recovered from insect collections using the second method. Seven isolates from blood samples were characterised as belonging to the BEF virus group from samples collected in the months of February, March, April and June 1990 and from April in 1991. One of these isolates, from March 1990, was neutralised by antibodies to Berrimah virus in the micro neutralisation test.

Discussion

The study of BEF virus group infections in Indonesian livestock was commenced because clinical disease in cattle is known to occur. It was considered important to clarify the ephemeral fever status of Indonesia and to develop laboratory diagnostic capacity.

The BEF-group viruses studied serologically were those available as type antigens together with type antisera from an Australian laboratory. However, other related virus may also be present. For example,

Malakal virus which was isolated from *Mansonia uniformis* in the Sudan, and Puchong virus, also isolated from these mosquitoes in Malaysia (Calisher et al. 1989). Other, undescribed, BEF-group viruses may also be present.

Serological results must be interpreted with care, because cross reactions among BEF-group viruses occur (Cybinski 1987). Hence antibodies to one BEF-group virus may be the result of infection with another BEF-group virus. None the less, the study of antibody prevalence in different species showed differences between viruses. All viruses tested were already known to infect cattle and buffalo in Australia on the basis of detection of antibodies (Cybinski and Zakrzewski 1983, Gard et al. 1984, Walker and Cybinski 1989). Losos (1986) reviewed the host range of BEF virus, and concluded that the virus does not naturally infect sheep, although they seroconvert when infected experimentally. No evidence of infection has been reported in goats, pigs or horses. However, after an epidemic of ephemeral fever in Taiwan, 1 of 16 sheep (6%) and 22 of 46

Table 4. Seroconversions to BEF virus in four groups of sentinel cattle at Depok, West Java.

Year	No. of cattle	Seroconversion (%)	Month of seroconversion
1987–1988	10	8 (80)	Jan, Feb, Mar, Jun, Jul
1988–1989	6	5 (83)	Dec, Mar, May, Sep
1989	7	4 (71)	May, Jun
1989–1990	4	2 (50)	Jun, Jul

Table 5. Insects collected at Depok, West Java, in 1991 and sorted according to species.

Insects	Genus/subgenus/group[1]	Species identified
Culicoides	Avarita subgenus[2]	*C. actoni*
Culicoides	Avarita subgenus[2]	*C. brevitarsis, C. dumduni C. flavipunctatus, C. fluvus, C. jacobsoni, C. orientalis* and *C. wadai*
Culicoides	Hoffmania subgenus	*C. insignipenis, C. peregrinus* and *C. sumatrae*
Culicoides	Trithecoides[2]	*C. albibasis, C. barnetti, C. gewertzi, C. palpifer* and *C. parahumeralis*
Culicoides	Meijerehelea subgenus	*C. arakawae* and *C. guttifer*
Culicoides	Shermoni group	*C. geminus*
	Clavipalpis group	*C. huffy*
	Schultzei group[2]	*C. oxystoma*
	Shortii group	*C. shortii*
Mosquitoes	*Aedes* spp.[2]	Not sorted further
	Anopheles spp.[2]	Not sorted further

[1] Wirth and Hubert (1989)
[2] Pools processed for isolation of viruses

goats (48%) had antibodies to BEF virus although the serological test protocol used was not specified (Chiu and Lu 1986).

Berrimah virus and Kimberley virus both differed from BEF virus in that antibodies were detected in small ruminants; sheep and goats in the case of Berrimah virus and goats in the case on Kimberley virus. In addition, antibodies to Kimberley virus were found in horses. A previous study in Australia failed to detect antibodies to Kimberley virus in sheep, goats and horses (Cybinski and Zakrzewski 1983). In this study, antibodies to Adelaide River virus were not found in cattle, and in only one buffalo. However other data (see Daniels et al. these proceedings) describes antibody to this virus in large ruminants in Indonesia. Goats had a higher seroprevalence for Adelaide River virus than the other viruses tested. The first report of Adelaide River virus (Gard et al. 1984), found antibodies in pigs but not in horses and goats. Clinical disease and serological reactors to BEF-group viruses in chickens and ducks have not been reported previously, and in this study there was no evidence of antibody in these species. Poultry would therefore not be useful as sentinel animals for BEF-group arboviral infections. Although the problem of serological cross reactions precludes definite interpretation of these results in a range of species, it appears that in a tropical endemic situation, Indonesia, the mammalian host range of the BEF-group viruses may be wider than previously indicated.

A sentinel animal program was considered important to allow opportunities for isolating viruses, and to start to describe the seasonal pattern of infections (Daniels et al. 1991). Depok in West Java was chosen as a high rainfall, low altitude site with a large cattle population, close to laboratory facilities. Seroconversions were observed from early in the wet season through to the end, from December to July. This pattern was somewhat different from that observed for bluetongue viral infections (Sendow et al. 1988), where the same group of sentinel animals in the two years 1987 to 1989 seroconverted to the bluetongue group orbiviruses predominantly in February and March every year. This may suggest that the vectors for these two groups of viruses differ, with some vectors of BEF virus being active earlier in the wet season. Preliminary analyses of antibodies to flaviviruses in these same calves in the 1987–1988 wet season also showed peaks of infection early in the wet season, from December to February (Sendow et al. 1988). In Indonesia flaviviruses have been isolated from *Aedes* spp., *Culex* spp. and *Anopheles* spp. mosquitoes.

Insect collections were made as a preliminary study of potential vectors, using insect identification and virus isolation from insects. BEF virus has been isolated from *C. brevitarsis* and *Anopheles bancrofti* in Australia, and from a pool of *Culicoides* spp. in Kenya. Kimberley virus has been isolated from *C. brevitarsis* and the mosquito *Culex annulirostris*. It has been suggested that *Culex annulirostris* may be a major vector of BEF virus (Muller and Standfast 1986). Our insect collections showed that potential vectors such as *Culicoides* spp. of the Avaritia subgenus, especially *C. brevitarsis* and *Anopheles* spp. mosquitoes were present. *Culex* spp. mosquitoes were not caught in the light traps.

Seven virus isolates from West Java have been provisionally typed to the BEF virus group, but only one of these was neutralised by antisera to the four viruses studied, namely Berrimah virus. Further work in two-way neutralisation tests may confirm this as the first identification of a BEF-group virus in Indonesia. Of equal interest is that the other six isolates were not neutralised by antisera to any of the four type viruses, indicating that other BEF group viruses have been isolated.

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The Natural History of Ephemeral Fever in Kenya

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Abstract

Ephemeral fever occurs in cattle in Kenya across a wide range of ecological zones, from semi-desert to temperate highland grasslands. None of the indigenous wild ruminant fauna manifest any clinical sign of disease, although serum neutralising antibody has been detected in some species. Clinical disease occurs either sporadically, or in epidemics lasting 2–3 years when there is a high morbidity in cattle. There is an association with rainfall but this is not absolute. There are clear interepidemic periods, when no clinical disease is evident anywhere in the country.

CLINICAL ephemeral fever in cattle was recognised in Kenya in the early years of this century. The disease was reproduced in 1913 by the subinoculation of blood from an infected to a susceptible bovine host at Kabete (Annual Report, Kenya Department of Agriculture, 1912–1913). The disease reappeared at intervals, usually in those cycles of wet years when Rift Valley fever was also encountered (Davies et al. 1985), and has continued intermittently ever since. Ephemeral fever was also reported in Uganda and Tanzania over the same periods (Annual Reports, Veterinary Departments, Uganda and Tanzania).

In indigenous zebu cattle (*Bos indicus*), the clinical signs of ephemeral fever are mild and do not persist for more than 1–2 days. In contrast, the disease in imported *Bos taurus* breeds is more severe and epidemics of ephemeral fever cause significant economic losses in milk production and depressed growth rates in beef cattle. While farmers are well aware of the effects of ephemeral fever on production, there are many other diseases which are considered to be of greater importance (rinderpest, Rift Valley fever, pleuropneumonia, East Coast fever, etc.), and little attention has been given to the problem of ephemeral fever in East Africa. This paper summarises the work on ephemeral fever carried out by the author from 1968 to 1989, at the Veterinary Research Laboratories, Kabete.

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Virus Isolation

In 1972 and 1973, bovine ephemeral fever (BEF) virus was isolated from clinical cases in the Rift Valley (altitude 1600-1800 metres, 0° 28S; 36° 13E) in semi-arid country, defined as Ecological Zone IV in the classification of Pratt et al. (1966). At that time there were local outbreaks of ephemeral fever, involving more than 1000 cattle of *Bos taurus, Bos indicus* and their crosses. The outbreaks were apparently not associated with any local rainfall, but the farms were adjacent to a fresh water lake (Naivasha) and a salt water lake (Nakuru).

Blood was collected, in EDTA anticoagulant, from clinical cases on the first day when signs were evident. Samples were transported on ice to the laboratory where the buffy coat was separated and each sample inoculated into a least four litters of 1–4 day old mice by the intracerebral route (Davies and Walker 1974) within one hour of returning to the laboratory. Some samples produced neurological signs on first passage in mice, but other isolations required two passages, and one isolate required four passages, before neurological signs became evident. In this initial study, BEF virus was isolated from at least half of the samples processed.

To reproduce clinical disease, two buffy coat samples from which virus had been isolated in mice, were inoculated into susceptible cattle. This resulted in clinical disease in one animal but only transient clinical signs in the other. Pre- and post-infection serum samples were collected for serology. Mouse ascitic fluids were also prepared from an Australian BEF virus isolate and from a Kenyan isolate,

K/86/73. These sera were used in neutralisation tests, initially in mice, and later in cell cultures.

In two-way, cross neutralisation tests (in infant mice and using pre- and post-inoculation cattle sera and ascitic fluids) no differences were evident between the Kenyan and Australian BEF viruses. Later, constant-virus varying-serum, microtitre neutralisation tests using cell cultures confirmed that the isolates from the two continents appeared to be antigenically identical.

Mammalian Host Range

There is relatively little information on the susceptibility of wild ruminants to BEF virus although the Asian water buffalo *Bubalus bubalis* has been shown to be mildly susceptible to ephemeral fever (Topacco et al. 1937). It is possible that BEF virus may have circulated as an inapparent infection amongst wild ruminant species and was undetected until susceptible cattle were introduced and clinical disease became evident. In order to establish whether BEF virus infection of wild ruminants occurs, sera were obtained from wild ruminants in the Rift Valley (Davies et al. 1975) and tested for the presence of neutralising antibody to BEF virus using a cell culture, microtitre neutralisation test with the K/86/73 isolate of BEF virus.

Fifty-four per cent of sera from African buffalo (*Syncerus caffer*) and 61% of sera from waterbuck (*Kobus ellipsiprymnus*) had neutralising antibody to BEF virus, wheras only 9% of wildebeest (*Connochaetes taurinus*), and 3% of hartebeest (*Alcelaphus buselaphus*) sera had antibody to BEF virus. No antibody was found in sera from impala, Grants or Thomsons gazelle, even though these species were very common in the ecological zone examined. Antibody could not be detected in sera from eland or oryx, although very few sera from these two species were examined. Infection of African buffalo seems to occur frequently and it is not uncommon to see titres of 1/80–1/320 in sera from these animals.

Ecological Range

A template for the classification of East African rangelands was made by Pratt et al. (1966), to relate the potential for livestock use with vegetational and moisture characteristics. This classification is also relevant to insect ecology. A summary of the principal features is given by Davies et al. (1975).

To investigate the distribution of BEF virus in Kenya, sera were collected from cattle at 36 sites, representing the whole range of ecological zones in Kenya. The cattle sampled had been exposed to an epidemic of ephemeral fever in 1967–1968 which spread throughout the whole country. Sera with neutralising antibody to BEF virus were found in all the ecological zones, from desert and semi-desert to the tropical coastal zone and temperate high altitude grasslands. In individual herds, seropositive rates of 24–95% were found, and high rates were often found in the drier zones. The results show that BEF virus infection can occur with a high frequency in all the ecological zones present in Kenya.

Interepidemic Periods

There have been periods when clinical disease appeared to be totally absent from any part of the country. Such a period was from 1969 until 1972, during which time surveillance for clinical disease was maintained over the whole country. In addition, active weekly examination was made of a sentinel herd located on site at Kabete farm and of other cattle at the same farm, a total of about 5000 animals. Only five seroconversions were detected in 1971 in the sentinel cattle.

An isolated outbreak of ephemeral fever, which was confirmed serologically, occurred in Ecological Zone V (dry thorn bush country) after local very heavy rains. The first cases were seen 10–12 days after the rains, followed by many more cases after 18–21 days. The majority of insects trapped at the site were *Culicoides* spp. and only 15% of the total light trap catch were mosquitoes.

To further investigate the role of wild ruminants in the ecology of ephemeral fever, sera collected from wild ruminants were examined for antibody to BEF virus. More than 50% of sera from buffalo and waterbuck born in an interepidemic period (1968 to 1972) had antibody to BEF virus, suggesting that virus infection of wild ruminants occurred when there were no reported cases of ephemeral fever in cattle. Unfortunately sera were not collected from cattle in that area during the same period. However it is interesting to note that the 1972–1973 outbreak of ephemeral fever occurred in an area where there was a high frequency of infection of buffalo in preceding years.

Vector Studies

BEF virus was isolated from a large mixed pool of *Culicoides* spp. collected at the site where BEF virus was isolated from cattle in 1972–1973. The insect isolate was from a mixed pool consisting of *C. kingii* 67%, *C. nivosus* 24%, *C. bedfordii* 8%, *C. pallidipennis* 1% and *C. cornutus* 1% (Davies and Walker 1974). However, virus was not isolated from nine other *Culicoides* pools, nor from five mosquito pools collected at the same site.

No BEF virus isolates were made from the many pools of *Culicoides* spp. processed over a period of

years when clinical ephemeral fever was not reported (Davies et al. 1979). In addition, BEF virus was not isolated from a large series of mosquito pools which were collected during interepidemic periods, even though many other viruses were isolated from this material (Linthicum et al. 1985).

General Observations

An hypothesis was made early in our ephemeral fever studies that if the virus was transmitted only by a mosquito vector, then the distribution of the disease should coincide with that of the vector. Furthermore, the distribution of ephemeral fever should be similar to that of other recognised mosquito transmitted diseases such as Rift Valley fever. As mosquito species are only found in certain ecological zones in Kenya (Davies et al. 1975) then ephemeral fever should be restricted to these zones and should not be widespread. The disease proved to be widespread, with many positive sera in semidesert areas where bovine antibody to Rift Valley fever virus was rarely detected. *Culicoides* spp. were also found to be widely distributed and were invariably present when outbreaks of ephemeral fever occurred. The sentinel herd of cattle at Kabete provided evidence that most cattle seroconverted to Akabane virus (Davies and Jesset 1985) and to many strains of bluetongue virus (Davies 1978) by the time they were two years of age. Both of these viruses are transmitted by *Culicoides* spp. and it is apparent that infections with these viruses was occurring every year. However, this was not the case with BEF virus at this site, as no seroconversions were detected in sentinel cattle during many of the study years when seroconversions to Akabane and bluetongue viruses occurred. If BEF virus is transmitted exclusively by *Culicoides* spp. then it must have a lower incidence in the vector population, much lower than bluetongue or Akabane viruses.

Another explanation might be that the virus is transovarially transmitted by mosquitoes, in the same manner as Rift Valley fever virus (Linthicum et al. 1985), and emerges when the floodwater breeding species are hatched following heavy and prolonged rains. A situation where *Culicoides* spp. could also be involved, as the virus is amplified in vertebrate hosts, then becomes possible. St George and Standfast (1983) have also drawn attention to this possibility as a result of their work in Queensland. Their data show that ephemeral fever oubreaks correlate more closely with increases in numbers of mosquitoes than with *C. brevitarsis* numbers.

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The 1990–1991 Epidemic of Ephemeral Fever in Egypt and the Potential for Spread to the Mediterranean Region

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Abstract

Clinical ephemeral fever occurred in Egypt in 1990–1991 and spread throughout the whole of the Nile Valley and Delta. High morbidity (20–90%) occurred in imported *Bos taurus* dairy breeds. Fattening cattle of local breeds in intensive systems were also severely affected. The disease was less severe in unimproved village animals. Water buffalo (*Bubalus bubalis*) were mildly affected at a low prevalence. The disease was economically significant due to loss of milk production and mortality in dairy and beef animals. Infertility problems were also important. The potential for further extension of ephemeral fever into North Africa and into some Mediterranean countries is discussed.

EPHEMERAL fever has been known to occur in Egypt for many years (Piot 1896, 1909; Rabagliati 1924). Ephemeral fever has occurred throughout most of Africa to the south of Egypt, where it is endemic with periodic epidemics. In the Middle East, the disease has only been recognised in epidemic form in Israel, Iran, Iraq and Saudi Arabia. There are unconfirmed reports that ephemeral fever may have occurred in the Eastern Mediterranean region during the period 1989–1991. The 38 °N latitude appears to be an ecoclimatic barrier as the disease has not been reported north of this latitude (Tanaka and Inaba 1986) except in China where it was reported at 44 °N (Zhang et al. these proceedings). Clinical cases of ephemeral fever have not yet been reported in Europe, although antibody to bovine ephemeral fever (BEF) virus has been detected in serological surverys in southern Russia.

Epidemiological Observations

Distribution of clinical cases

Clinical ephemeral fever was reported in 1990 and 1991 from most of the Governates in Egypt. The Governates of Aswan, Quena, Sohag and Assiut in Upper Egypt were first affected in the summer of 1990. Further cases were described later that year from El Minya and Beni Suef. The disease was comparatively mild and occurred mainly in indigenous cattle and in buffalo as few exotic cattle were kept in those areas. Damietta in the eastern part of the Delta was affected in the autumn of 1990. In 1991 the Delta Governates experienced the disease, as well as all the Nile Valley Governates and the oases of Fayoum, Bahariya, Dakahlia and New Valley. In rice growing areas, morbidity rates appeared to be higher and cattle appeared to be more severely affected.

Epidemic pattern

Ephemeral fever occurred in the irrigated lands along the Valley of the Nile, throughout the Nile Delta and the more recently cultivated areas at the edge of the Delta. The desert oases far removed from the Nile Valley also reported the disease, as have well-head irrigation schemes. This suggests that aerial movement of the virus occurred. Clinical cases occurred in both summer and winter months. Although the prevalence of the disease was higher in June to August and fewer cases occurred in the winter months, the disease did not totally disappear in winter as has been described in some temperate zones of southern Africa.

Host Range

Domestic cattle, both indigenous and exotic to Egypt, were clinically affected. Imported Holstein cattle were more severely affected than native breeds (mostly *Bos taurus*) which showed milder clinical

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signs of shorter duration, and had lower production losses. Morbidity rates in individual herds of imported cattle varied from 20-90%. Mortality rates in imported cattle were in the range 1.5-3%. The animals most likely to die were the largest, heaviest and highest producers. Thus the most valuable animals in the herd appeared to be the most susceptible to ephemeral fever.

Many indigenous animals which would have recovered were slaughtered in the early months of the epidemic, when they became recumbent. Other diseases which stock owners are familiar with, such as Rift Valley fever, and which cause prostration, often result in death. However, farmers soon became more familiar with ephemeral fever, where recovery was rapid and often complete, and fewer animals were slaughtered. Similar responses by farmers to outbreaks of ephemeral fever have also been reported from Indonesia (see Daniels et al. these proceedings).

The morbidity rate in buffalo was much lower than in cattle with only about 5% of buffalo showing any clinical signs. Very mild, transient and presumably subclinical infections occurred and there was little apparent fall in milk yield.

Age groups affected

Calves, weaners and young animals up to two years of age were generally far less severely affected than other age groups and cattle 2-3 years old showed only mild clinical signs. The most severe signs were seen in large, heavy milking cattle of Friesian or Holstein type, of more than 3-4 lactations, when they were in heaviest production. Large dairy bulls were frequently very severely affected.

Economic Effects

Farms with imported Holstein cattle and their crosses reported a 50% decrease in milk production over the outbreak period. The outbreaks lasted for 6-12 weeks in individual herds. Production then increased, but only to 75-85% of the expected yield.

Up to 80% of cattle in intensive feedlot systems developed clinical disease which was milder in the indigenous breeds than in the imported breeds. Heavier animals experienced more severe disease than lighter animals. Weekly weighing showed either no weight gains or loss of weight for periods of 3-6 weeks or longer after the onset of clinical signs.

Abortion rates of up to 3% were reported in Holstein cattle, usually at 4-7 months gestational age. This was probably an indirect effect as BEF virus does not infect the foetus. Detailed analysis in one herd with 80-90% morbidity showed 30% loss

of foetuses of 45-105 days gestation, and some 10% loss of foetuses of 105-290 days. Laboratory tests ruled out the possibility of intercurrent brucellosis, vibriosis and trichomoniasis. Some bulls which had ephemeral fever were found to be infertile during the epidemic.

General Observations

There was a countrywide epidemic of ephemeral fever in Egypt in 1991 affecting approximately 250 000 imported cattle and a smaller number of indigenous cattle and buffalo. The disease produced its most severe clinical and economic effects in the intensive, highly productive milk and beef fattening sectors of the cattle industry. The intensive milk producing farms have a disproportionate importance in Egypt, for they supply fresh milk to the urban populations of Cairo and other cities. The rural population consumes mostly buffalo milk, and these animals were much less severly affected than imported cattle.

The disease may have entered Egypt from the south or from the east by the aerial movement of arthropod vectors. The vectors of BEF virus in Egypt have not yet been identified, but mosquito and *Culicoides* species are abundant, the former especially so in rice-growing areas.

Potential for further extension of ephemeral fever in the region

There have been a number of studies of the movement of insect pests and insects capable of acting as vectors for BEF virus in the Middle East and Eastern Mediterranean region (Williams 1924; Rainey 1951, 1973; Garret-Jones 1962; Dinoor and Levi 1967; Sellars 1980; Shimshony et al. 1989).

Air currents have been shown to be capable of carrying insects north from sub-Saharan Africa. These insects might be expected to to carry BEF virus and other arboviruses into Egypt, Israel, Lebanon, Iran, Iraq, Syria, Turkey, Saudi Arabia, Arabia, Yemen and the Arabian peninsula. Ephemeral fever has already been described in most of these countries even though it is not a notifiable disease under the regulations of the OIE.

The potential also exists for the periodic windborne spread of BEF virus to countries such as Yugoslavia, Bulgaria, Albania, Italy, Cyprus, Greece and possibly even Spain, as well as to North African countries. A knowledge of the distribution of potential vectors of BEF virus in these countries would be valuable and surveillance should be maintained when there are epidemics in the Middle East.

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Epidemiology, Clinical Findings and Treatment of Ephemeral Fever in Buffalo (*Bubalus bubalis*)

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Abstract

This paper describes the epidemiology, clinical findings and treatment of ephemeral fever in buffalo (*Bubalus bubalis*) in Gujarat State, India where the disease is now endemic. The occurrence of the disease has changed from the previous pattern of severe sporadic epidemics, to a slow moving moderate epidemic, with about 10% morbidity and 1–2% mortality. Most cases occur in the summer and monsoon seasons, with occasional cases during the remainder of the year. The disease is of considerable economic importance due to a drastic reduction of up to 70% in milk production and the death of a few animals. Factors which appear to contribute to the disease include: a sudden change of weather; increase in the insect population; advanced pregnancy; parturition and high milk production. The disease is more common in well fed, healthy buffaloes. In most cases the disease is subacute. There is a sudden high temperature, anorexia, a sharp fall in milk production, profound dullness and depression, shivering, stiffness and lameness in one or more limbs. Clinical signs last for 4–5 days followed by spontaneous recovery. Some cases become complicated and finally die. Most cases recover spontaneously or with simple treatment. The acute cases respond well to calcium gluconate, dextrose, vitamins, analgesics and phenylbutazone injections. Good nursing care helps in early recovery.

BOVINE ephemeral fever (BEF) is a disease of cattle and buffalo caused by an insect-borne rhabdovirus. The disease is characterised by inflammation of mesodermal tissues and manifested by muscular stiffness, lameness etc. (Blood and Radostits 1989). Ephemeral fever in buffalo has only rarely been described in other countries. This paper reports general field observations on ephemeral fever in buffaloes (*Bubalus bubalis*) in Gujarat, India.

Epidemiology

Ephemeral fever in Gujarat now occurs endemically with occasional outbreaks. However in previous times the disease used to occur as sporadic, severe epidemics. This change from epidemic to endemic behaviour has been observed in cattle in other countries (Blood and Radostits 1989). Compared to the situation in cattle, published reports of clinical disease in buffalo are infrequent. The experimental

infection of buffalo with BEF virus has been reported by Young (1979).

Moderate attacks occur annually during late summer and monsoon season, with occasional cases at other times of the year. Mild cases are often not reported as owners do not need the services of a veterinarian. Ephemeral fever causes considerable economic loss due to a drastic transient reduction of about 70% of milk production and death of a few severely affected animals. Milk production often fails to return to normal level until next lactation (Fraser et al. 1986).

A number of predisposing factors appear to lead to the occurrence of the disease. They include: a sudden change of weather from hot summer to monsoon; an increase in insect numbers; and stress factors such as advanced pregnancy, parturition, high milk production. The disease occurs more commonly and more severely in well fed, healthy adult buffaloes.

Clinical findings

The symptoms of ephemeral fever in buffaloes are the same as in cattle. In most cases, the disease is

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subacute. There is sudden rise of body temperature (105–106 °F), complete anorexia, sharp fall in milk yield, profound dullness and depression, constipation, ruminal stasis, drooling salivation, dry muzzle, nasal and ocular discharges, muscle shivering, weakness of limbs and lameness in one or more limbs. Some buffaloes also show respiratory signs. On the second day clinical signs are more severe than on the first day. There is more pronounced stiffness, weakness, lameness and animals adopt a posture similar to that of acute laminitis, with all four feet placed well under the body. The animal is reluctant to move and prefers to lie down quietly most of the time. Some animals are unable to get up, even on application of pain stimuli and adopt a posture similar to that of parturient paresis, that is, a sternal recumbency and head turned in to the flank. On rising most animals become recumbent again in a few minutes as they are unable to bear weight. The shifting of pain from one limb to another is characteristic.

On the third day there is usually little or no improvement. Occasionally, very severe cases remain down and adopt a posture of lateral recumbency. About the fourth day, the animals start eating and ruminating, their body temperature decreases, and they are able to rise alone showing moderate improvement. Most of the cases recover rapidly on the fifth day and completely recover on the sixth or seventh day.

Occasionally, cases of long duration develop subcutaneous emphysema, bed sores and ulcers, maggot infestation, show downer cow syndrome and finally die. Subcutaneous emphysema has been observed by Theodoridis and Coetzer (1979). Occasional cases become complicated with abortion due to high fever. Mild clinical cases occur at the end of outbreak with mild signs of fever, lameness and inappetence.

The characteristic clinical signs of fever, limb stiffness and pain are due to viral septicaemia which produce inflammation of mesodermal tissue especially joints, lymph nodes and muscles (Burgess 1971).

Treatment

Most cases recover spontaneously or with simple treatment. Prompt adequate treatment is necessary to get early recovery, to regain lost production and to avoid complications and losses. A successful treatment used in Gujarat is intravenous 20% dextrose and analgesics together with intramuscular phenylbutazone and multivitamins. Two such treatments on consecutive days are usually sufficient. Recumbent cases respond well to intravenous calcium gluconate. Good nursing care helps in early recovery. In acute cases oral medication is to be avoided. In an experimental study on the effect of phenylbutazone for the prevention of ephemeral fever, Uren et al. (1989) observed that the drug prevented fever and other clinical signs in 6 out of 16 cattle.

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Pathology, Pathogenesis and Diagnosis

The papers included in this section reflect the present state of knowledge on the pathology of ephemeral fever. Although the disease is very serious in economic terms, the pathology has not been well described. The evidence presented in these papers confirms that it is inflammatory in nature. There is also strong support for this from biochemical indicators and the success of anti-inflammatory treatments (which is unusual for a viral disease). However, the pathologists have so far been unable to determine why fatalities occur and the site of viral multiplication has not been identified.

As the primary effects of bovine ephemeral fever virus are in the vascular system, investigations need to be focused in this tissue to identify the target cells. Improvements in technology, such as efficient virus isolation, electron microscopy and molecular probes, all increase the chances of success.

Pathological Changes in Cattle Experimentally Infected with Bovine Ephemeral Fever Virus

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Abstract

Ninety-three cattle experimentally infected with the Chinese (Beijing 1) strain of bovine ephemeral fever virus were necropsied 2-10 days post-infection. The principal pathological changes observed were serofibrinous arthritis (59% of cases), interstitial emphysema (31%), nephritic infarcts (69%) and bronchopneumonia (9%). The severity of the lesions appeared to be greater in cows than in steers.

BOVINE ephemeral fever (BEF) is characterised by sudden illness and fever. Mortality is usually very low although the morbidity can be high. Because of the low mortality, field necropsies are infrequently undertaken and there is relatively little information available on the pathology of the naturally occurring disease. Some studies have been undertaken on the pathology of the experimental disease in other countries (Basson et al. 1970; Young and Spradbrow 1990). Experiments were carried out to reproduce the disease with a Chinese isolate of BEF virus and to describe the pathology of these experimental cases.

Materials and Methods

In all, 93 cattle (16 cows and 77 steers) were used. The ages varied from one to five years and all were proven to be free of ephemeral fever prior to experimental infection. The animals were infected by intravenous injection of 1-5 mL of the Chinese (Beijing strain) of BEF virus. Clinical signs usually appeared 3-5 days after viral inoculation and the animals were killed 2-10 days after the cessation of fever. Tissue samples were fixed with 10% formalin and then embedded in paraffin. Sections were cut, stained with haematoxylin and eosin and examined microscopically.

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Results

Interstitial emphysema was observed in the lungs of a large number of animals (31.5%) and was observed mainly in the apical lobes and the anterior surface of the cardiac and diaphragmatic lobes. Lung parenchyma exhibited congestion, oedema and overdistension of alveoli. Haemorrhagic changes occurred in a few cases. Dark red, and dull purple liver-like lesions, 1-3 cm in diameter, were observed in the lung parenchyma of 9% of cases. Interstitial spaces were thickened in a number of animals. Histological examination revealed a large amount of mucus, fibrin and dead epithelial cells in bronchi as well as swollen ciliated epithelial cells. The submucosal tissues were infiltrated by a large number of lymphocytes and the alveolar spaces were reduced or obliterated. The walls of small arteries were thickened, the endothelial cells were swollen, and in some cases, the lumen was totally occluded.

In 59% of cases there was swelling of the shoulder, knee or hock joints and the volume of joint fluid was increased. In the early stages of the disease, the joint fluid was transparent or only slightly turbid but as the disease progressed the joint fluid became cloudy and grey. In 44% of cases there were fibrin flakes in the joint fluid. In later stages of the disease, fibrin was attached to the articular surfaces and joint capsules. In two cases there was blood in the joint fluid and in a third case, there was an ulcer on the articular surface. Fourteen of the 16 cows examined had a severe serofibrinous arthritis.

In 69% of cases, the kidneys were swollen and the cortex pale. Grey-white necrotic foci, varying in size

and number were found in 43% of the cases. The necroses in the kidney surface were wedge shaped. Petecchial haemorrhage was seen in one case and cortical micro-abscesses in another. Histologically the arterial walls were thickened and endothelial cells were swollen, hyperplastic and sloughing. The lumen of some arteries was completely blocked. Focal necrosis, typical of nephroanaemic infarct, was seen in the kidney cortex.

Livers were slightly swollen and fragile in 35% of cases. Histological examination showed granular degenerating hepatocytes, swollen Kupffer cells and hepatic sinuses infiltrated with lymphocytes.

In 13% of cases, the endocardium had striped or spotted haemorrhages and the mycocardium was soft and light coloured. Histological examination revealed granular degeneration of the mycocardium, and in some cases, lymphocytic infiltration around the cardiac blood vessels.

The spleen in 55% of cases exhibited swelling of the pulp and indistinct splenic trabeculae. In 11% of cases which had a prolonged clinical course, a slight hyperplasia was seen in the splenic follicles. Microscopic examination revealed that the endothelial cells of splenic central arterioles were swollen, hyperplastic, sloughing and occluding the lumen. Basilar membrane cells were swollen, resulting in a thickened arterial wall. Most of follicles were ruptured or atrophic, while a few of the germinal centres in follicles were enlarged.

Lymph nodes, especially the cervical and popliteal, were enlarged. Focal haemorrhage was seen in eight cases and focal cortical necrosis in two cases. Microscopical examination revealed that the endothelial cells of small arteries were swollen and hyperplastic and there were large numbers of lymphocytes in the sinuses. Some germinal centres in the lymph nodes were slightly enlarged. Some reticular cells exhibited proliferation and some showed degeneration and necrosis.

The tonsils of most cases were swollen and yellowish. Microscopically, focal necrosis was observed in the epithelium of the crypts and the follicles were swollen. Reticular cells in the germinal centres were also swollen and necrotic. In three cases focal haemorrhage was observed in the laryngeal mucosa.

Discussion

The main pathological features in cattle experimentally infected with bovine ephemeral fever virus were serofibrous arthritis, bronchopneumonia, interstitial emphysema and nephronecrosis. The frequency and the severity of pathological lesions appeared to be more pronounced in cows than steers, although only 16 cows were infected, compared to 77 steers. The pathological manifestations in cattle inoculated with Chinese bovine ephemeral fever virus strain were similar to those observed by Basson et al. (1970) and by Young and Spradbrow (1990), in which the main pathological changes occurred in joints and muscles.

A comparison of our observations of the pathology of experimental and natural cases revealed that haemorrhagic and enteric tract changes were more obvious and serious in natural cases. Whether this resulted from coinfections is difficult to determine.

By histological examination, the main pathological change observed in our experimental cases was damage to small arteries throughout the body, resulting in endothelial cells becoming swollen, hyperplastic and detached, eventually blocking the lumen. The main effect of this damage appeared to be disruption of the circulatory system with poor nutrition of tissues and the appearance of focal necrosis. The pathological changes may indicate that BEF virus has an affinity for endothelial cells and virus replication may cause direct damage to the cells.

Similar pathological changes were observed in the blood vessels of lymph nodes and spleen, however there did not appear to be any serious damage to the immune cells. Animals killed eight days after cessation of fever had enlarged germinal centres and lymphocyte proliferation. This phenomenon indicated an increase in immune activity, which is possibly the main reason for the low mortality of this disease.

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The Pathology of Bovine Ephemeral Fever with Special Reference to the Pathogenesis of the Joint and Skeletal Muscle Lesions and Pulmonary Emphysema

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Abstract

The pathology of ephemeral fever has been reviewed with reference to the pathogenesis of the disease. The main lesions are found in joints, periarticular tissues, tendon sheaths and certain skeletal muscles of locomotion but the vascular, genital, respiratory and central nervous systems may also be affected. In typical cases of the disease, vasculitis, sometimes accompanied by thrombosis, is associated with serofibrinous synovitis and fasciitis and focal necrotic muscle lesions. The presence of numerous neutrophils, particularly in the affected joints and tendon sheaths, and high levels of interferon would seem to influence the severity of these lesions.

Bronchiolitis is thought to play a role in the development of pulmonary and subcutaneous emphysema which is sometimes present in cattle suffering from the disease. In animals that have been recumbent for a prolonged period, status spongiosus of certain white matter tracts in the spinal cord and brain have been reported. Sperm abnormalities and an increase in the somatic cell count in the milk occur in affected animals.

SINCE the first recognition of bovine ephemeral fever in Zimbabwe by Bevan (1907), surprisingly few studies have been carried out on the subcontinent. The only detailed report on ephemeral fever in the region is by Basson et al. (1970) describing joint and muscle lesions in experimentally produced cases. Although outbreaks of ephemeral fever occur almost annually in South Africa and in other countries throughout the world, few cases are presented for routine necropsy with the result that the pathology of the natural disease is poorly described. The aim of this paper is to review the pathology of ephemeral fever and to discuss the pathogenic mechanisms which play a role in the development of lesions.

Pathology

Joint, periarticular, tendon sheath and skeletal muscle lesions

Typical clinical signs of ephemeral fever such as stiffness, lameness, paresis and recumbency are the result of inflammatory lesions in the joints, periarticular tissues, tendon sheaths and skeletal muscles. There is a correlation between the severity of the lesions and clinical signs. Basson et al. (1970) gave a detailed account of the joint, periarticular, tendon sheath and skeletal muscle lesions at 1–4 days, 6 days and 10–15 days after the febrile reaction in cattle infected experimentally with BEF virus. Most of the information reported below was obtained from that publication or from unpublished observations made by the authors, unless otherwise stated.

Lesions are invariably more severe in the limbs on which the animal is lame but there may be considerable variation in the severity of the joint lesions in the same animal. While two or three joints are usually severely affected, other joints may not be involved or may only show mild lesions. The inflammatory changes are usually most severe in the larger joints of affected limbs: the stifle, followed by the hip, shoulder and elbow joints with approximate

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equal frequency, are most constantly involved, while the carpal, tarsal and fetlock joints are less commonly affected. In severely affected joints, excessive amounts of turbid, straw-coloured fluid (in which small yellowish flakes are suspended) and large fibrin coagula are present. The synovial membranes are oedematous and contain petechiae but the articular cartilages are normal. In less severely affected joints, diffuse congestion and a few petechiae in the synovial membranes accompanied by a slight increase in the synovial fluid are usually evident.

Fasciitis characterised by the presence of small haemorrhages and accumulation of serofibrinous exudate in the loose connective tissue is most prominent in the periarticular areas of affected joints. The lesions in the tendon sheaths are similar to those described for the joints. Microscopically, joint lesions 1–4 days after the onset of fever are characterised by fibrinopurulent synovitis and an accumulation in the joint cavity of a copious exudate comprising fibrin, numerous neutrophils and a few mononuclear cells. The lining cells of the synovial membrane are destroyed where the exudate adheres to them. Other lesions include oedema, petechial haemorrhages and a mild infiltration of mostly neutrophils, with a few lymphocytes and plasma cells, particularly around small blood vessels in the synovial membrane and synovial layers of the joint capsules. The walls of some of these blood vessels are infiltrated by inflammatory cells and in many of them there is a leukostasis, consisting mainly of neutrophils. On day four after the onset of fever there is swelling of endothelium and hyperplasia of both endothelial cells and pericytes of blood vessels in the synovial membrane. From day six the exudate consists of large masses of coagulated fibrin containing few neutrophils or other cells, and in growth of fibroblasts and capillaries into the fibrin masses where the latter attach to the joint capsule.

At the same time a mild perivascular lymphocytic infiltration is present and swelling and severe hyperplasia of endothelium culminate in partial or complete occlusion of many venules, arterioles and capillaries. Perivascular fibrosis is particularly pronounced in the synovial membrane 10–15 days after the onset of fever.

Pale, well-circumscribed areas, one to a few centimetres in diameter are present in skeletal muscles particularly near the attachments of certain muscles. Petechiae or ecchymoses are often associated with these lesions which are most commonly found in the quadriceps group but other muscles including the longissimus dorsi, biceps femoris, triceps, semimembranosus and semitendinosus may also be affected. One experimental animal infected by Basson et al.

(1970) became paretic and remained recumbent for 10 days before it was killed. This animal showed conspicuous greyish areas of necrosis of almost all the muscles of all limbs and a severe cellulitis affecting the subcutis of the lower half of the limbs.

Localised serofibrinous fasciitis (sometimes accompanied by the presence of petechiae and ecchymoses) of the intermuscular connective tissue is most prominent in the large muscle groups of the fore and hind limbs and in the epimysium of some of these muscles. Localised serofibrinous cellulitis in the immediate vicinity of affected joints may be present.

Microscopically the muscle lesions at 1–4 days after the onset of fever are characterised by hyaline degeneration and necrosis; varying sized focal haemorrhages; a mild infiltration of particularly neutrophils, a few mononuclear cells, and in some necrotic muscle fibres also small numbers of macrophages; and slight proliferation of sarcolemma nuclei. From day six large numbers of mononuclear cells (predominantly macrophages), are present in and between necrotic muscle fibres, and there is also some evidence of sarcolemma nuclei proliferation, fibroplasia and mineralisation of the sarcoplasm of remaining necrotic muscle fibres. Similar but less severe vascular lesions (sometimes accompanied by fibrinoid changes of the walls of small arteries and thrombosis of small blood vessels) to those described in the synovial membrane occur near necrotic foci in the muscles. Perivascular fibrosis may also be present.

Pulmonary lesions

Signs of respiratory involvement including increased respiratory rate and respiratory distress, sometimes accompanied by subcutaneous emphysema, are occasionally present in animals which manifest typical signs of the disease or which have shown no or little stiffness previously (Theodoridis and Coetzer 1979; McFarlane and Haig 1955). These signs and death are sometimes precipitated by forced movement of animals. Animals showing severe subcutaneous emphysema may however recover fully in 2–3 weeks (Erasmus et al. 1974).

In fatal cases, severe pulmonary emphysema is the most striking lesion. The emphysematous lungs occupy almost the entire thoracic cavity, do not collapse on opening the thorax, and bullae ranging from 2–10 cm in diameter are found in the septa, parenchyma and subpleurally. Areas of atelectasis occur adjacent to these bullae. Emphysema is also evident in the mediastinum and around the pericardium and subperitoneally along the ventral part of the vertebral column, around the kidneys, spleen and other abdominal organs and in the perirectal area.

Microscopically, the terminal and respiratory bronchioles reveal accumulation of large amount of cellular debris comprising numerous neutrophils, macrophages, erythrocytes and fibrin and sometimes also focal necrosis and infiltration of neutrophils of the mucosa, and focal hyaline degeneration and necrosis of the muscularis mucosae. Leukostasis of small blood vessels in close proximity to affected bronchioles and an infiltrate of some neutrophils and a few eosinophils, are frequently encountered in the peribronchiolar loose connective tissue. Large bullae are found in the lung tissue as a result of rupture of alveoli and bronchioles (Theodoridis and Coetzer 1979). The presence of an exudate in bronchioles has also been reported by Burgess and Spradbrow (1977).

Involvement of udder

It is well known that BEF virus may cause a severe reduction in milk production in cows (Henning 1956; Theodoridis et al. 1973; Davis et al. 1984) but its precise action on the udder is poorly understood. The effects of ephemeral fever on early, middle and advanced stages of lactation have been studied (Theodoridis et al. 1973). Milk production is reduced by an average of $59 \pm 22\%$ during the febrile period of the disease. Milk production of cows infected during early lactation returns to normal levels more readily than that of cows infected later, apparently because there is a tendency towards proliferative cellular activities during early lactation and involutive cellular activities during the later stages (Theodoridis et al. 1973).

The quality of the milk of acutely affected cows is altered. There is an increase in the total somatic cell counts (increased numbers of sloughed epithelial cells and mononuclear cells, but the numbers of neutrophils are reduced) and bacteria (such as *Streptococcus epidermidis, Streptococcus dysgalactiae, Pseudomonas aeruginosa, Staphylococcus aureus*) in the milk of some quarters of the udder. It has been suggested that the decreased numbers of neutrophils in the milk during the acute disease lower the resistance of the udder and predispose the animals to bacterial udder infections (Theodoridis et al. 1973).

Cows do not develop clinical mastitis, but according to international standards on somatic cell counts they can be diagnosed as having subclinical mastitis (Theodoridis et al. 1973). The quality of milk returns to normal 2-12 days after infection and there is no permanent damage to the udder; milk production during subsequent lactations is not affected.

Testicular involvement

Bulls may suffer from a temporary reduction in fertility for several weeks following acute clincal disease.

The cause of infertility has not been studied in detail, but a drastic rise of up to 74% (compared to a normal 3-10%) in mid-piece abnormalities has been reported, commencing within the first two weeks following acute illness and reaching a peak within 3-8 weeks (Chenoweth and Burgess 1972). A higher than normal percentage of abnormal spermatozoa may be found for up to 24 weeks after clinical disease. It is not known whether sperm abnormalities are caused by virus multiplication in the testes or merely the result of the febrile reaction.

Central nervous system lesions

Lesions are not usually present in the central nervous system during the acute phase. However, motor disturbances of the hind limbs (manifested as ataxia of the hind quarters or prolonged recumbency) for a few weeks to several months following infection (with or without clinical signs) have been reported in a small percentage of animals. The appetite of these animals is usually undisturbed (Armfield 1915; Rosen 1931; Gray 1938; Mackerras et al. 1940; MacFarlane and Haig 1955; Henning 1956; Spradbrow and Francis 1969; Basson et al. 1970; Snowdon 1970; Hill and Schultz 1977).

Bilateral symmetrical non-inflammatory degeneration of varying severity of one or more of the funiculi (ventral, lateral or dorsal) of the spinal cord have been reported (Hill and Schultz 1977). The authors noted that these lesions only occurred in the cervical and/or lumbar regions. However, similar lesions may also occur in the medulla oblongata and cerebellar peduncles. The lesions in the white matter are characterised by numerous vacuoles, swollen eosinophilic axons, and the presence of some macrophages containing myelin breakdown products. Due to the similarities of these lesions to those caused by compression of the spinal cord, it has been postulated that they might be caused by extreme flexion of the occipito- atlantal and atlanto-axial joints resulting in stenosis of the vertebral canal or stretching of the cord over the ventral edge of the foramen magnum and over the bodies of the first cervical vertebrae (Hill and Schultz 1977). Heavy animals suffering from acute ephemeral fever may be very ataxic, and may fall heavily which could result in trauma to the spinal cord.

Haematological changes

Apart from the inflammatory lesions in various tissues, significant haematological and biochemical changes have been reported in cattle suffering from the disease. Clinical disease is accompanied by a marked neutrophilia of $9.6-2.5 \times 10^9$/litre (Mackerras et al. 1940; St George et al. 1984) and

lymphopenia (Uren and Murphy, 1985). A decline of lymphocyte numbers, from a mean of 10×10^9/litre two days before the onset of clinical disease, to $5\text{--}7 \times 10^9$/litre on the day of the peak of the febrile reaction has been reported (Uren and Murphy 1985).

Plasma fibrinogen levels (normal values 6.0–8.0 g/litre) rise rapidly to maximum levels on the day after the febrile peak and then fall gradually over the next four days (St George et al. 1984). Serum calcium levels decline on the first day of clinical disease and reach lowest levels on the second day of disease, before returning to normal levels over the next four days (St George et al. 1984; Murphy et al. 1986; St George, in Press). The mean level of 2.13 mmol/litre is only slightly below the lower levels of the normal range of 2.25–2.75 mmol/litre (St George et al. 1984). The reason for the reduced calcium levels is not known, but rumen stasis and hypomotility of the digestive tract, by reducing calcium intake, may contribute to the reduction in calcium levels (St George et al. 1984).

Other lesions

Animals suffering from ephemeral fever may show a slight subcutaneous oedematous swelling around the eyes (Bevan 1912), stasis of the rumen and the intestinal tract, and slight enlargement of the lymph nodes of the limbs. Bloat, hypostatic or foreign body pneumonia may be evident in recumbent cases.

Pathogenesis of the Most Important Lesions

Bovine ephemeral fever virus antigen is found in neutrophils, mesothelial cells of serosal surfaces and epithelial cells of synovial membranes (Young and Spradbrow 1985). It has been suggested that BEF virus is not cytocidal and that neutrophils and high levels of interferon and substances mediated by it are important in the production of the inflammatory lesions (St George, in Press). A characteristic of the joint, tendon sheath and skeletal muscle lesions is the presence of large numbers of neutrophils, many of which contain viral antigen (Basson et al. 1970; Young and Spradbrow 1985). Neutrophils seem to influence the severity of clinical disease. Suppression of neutrophils results in much milder disease and it has been postulated that ephemeral fever may be an acute immune-complex disease (Young and Spradbrow 1980).

The vascular lesions are usually mild and occur in close association with lesions (Basson et al. 1970). However, vasculitis plays a central role in the development of the serofibrinous effusive lesions of the synovial membranes, tendon sheaths and fasciae and the focal necrotic muscle lesions (Mackerras et al. 1940; Basson et al. 1970). The inflammatory lesions in the tissues are accompanied by a two- to fourfold increase in plasma fibrinogen levels (Uren and Murphy 1985; St George et al. 1984).

Although the pathogenesis of the pulmonary emphysema is not fully understood, bronchiolitis conceivably results in rupture of bronchioles and alveoli and escape of air into the connective tissue septa and lymphatics of the lungs (Theodoridis and Coetzer 1979). From here the air extends subpleurally to the mediastinum and thoracic inlet to reach the subcutaneous tissues or passes posteriorly to the abdominal cavity and accumulates subperitoneally.

Apart from the inflammatory lesions in the joints, tendon sheaths and skeletal muscles, the reduction in the level of ionised and bound fractions of calcium in the blood and not central nervous system lesions, would seem to contribute significantly to the paretic or paralytic signs which are often present in acutely affected animals (St George et al. 1984; Murphy et al. 1986; St George, in Press).

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Bone Marrow: A possible site for Replication of Bovine Ephemeral Fever Virus

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Abstract

Bovine ephemeral fever virus was isolated from the bone marrow of three experimentally infected cattle at 42, 44 and 48 hours post-infection. Virus was not detected in peripheral blood leucocytes until 56, 66 and 66 hours respectively. The isolates were injected intravenously into four other seronegative bulls which also developed typical clinical disease and seroconverted. It is theorised that bone marrow may be one of the sites in which BEF virus replicates in cattle.

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The Relationship between Selenium Deficiency and the Development of Pulmonary and Subcutaneous Emphysema in Bovine Ephemeral Fever Virus-Infected Cattle

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Abstract

Bovine ephemeral fever (BEF) was diagnosed on several commercial farms around Harare, Zimbabwe. The affected animals showed signs of fever (40–41.5 °C), depression, rumina stasis, lameness and recumbency. Eight of those attended had severe respiratory distress and sub-cutaneous emphysema. Haematological and biochemical results indicated leucocytosis with an attending lymphopaenia. Selenium deficiency was detected only in those animals which showed respiratory embarrassment and subcutaneous emphysema.

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The Diagnosis and Differential Diagnosis of Bovine Ephemeral Fever in Southern Africa

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Abstract

The clinical signs of ephemeral fever are not sufficiently characteristic for a diagnosis to be made in individual animals. However a diagnosis can be made by considering epidemiological, clinical and pathological findings in association with specific laboratory techniques. In southern Africa, a wide variety of other infectious and non-infectious diseases could be confused with ephemeral fever, especially botulism, diplodiosis, acute bovine pulmonary oedema, emphysema and hypocalcaemic conditions. The differential diagnosis of these diseases is discussed.

BOVINE ephemeral fever (BEF) occurs regularly in many countries in Africa, Australia and Asia (St George et al. 1977; Uren et al. 1983). In South Africa, the disease is reported almost every year in some parts of the country. Epidemics were reported in 1955, 1966-68, 1974-78 and 1981-84. Outbreaks of ephemeral fever often occur concurrently with other infectious diseases, especially when climatic conditions favour the breeding of large populations of arthropod vectors. Although both *Culicoides* midges and mosquitoes have been incriminated as vectors of BEF virus for many decades, isolation of the virus from wild-caught insects has been achieved on only a few occasions and the definitive vectors have not yet been identified. BEF virus has not yet been isolated from insects in South Africa (Theodoridis et al. 1979).

The clinical signs caused by infection with BEF virus are not characteristic and may be confused with other diseases which cause lameness or paresis. The Afrikaans term used for ephemeral fever, *drie-dae-stywesiekte* (three-day-stiff sickness), is also used to describe two other conditions. One is caused by the plant *Crotalaria burkeana* (stywesiektebossie) which gives rise to laminitis and abnormal outgrowth of the hooves. The other condition is an aphosphorosis syndrome characterised by osteomalacia of the long bones of the limbs.

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The aim of this paper is to highlight the most important features of ephemeral fever and to describe techniques which should be considered in making a diagnosis of ephemeral fever. Attention is also given to diseases occurring in southern Africa which must be considered in the differential diagnosis.

Diagnosis

Because ephemeral fever is an insect transmitted disease, it has a seasonal occurrence in temperate climates, most cases being reported in late summer and autumn. Outbreaks of ephemeral fever usually terminate soon after the appearance of the first frost and a diagnosis of the disease is thus most unlikely in mid-winter or early spring when vector numbers are low. However, in tropical and subtropical areas, the disease can occur at any time of the year.

The clinical signs of ephemeral fever in cattle are very variable and individual cases may easily go unrecognised. Typically, infected animals present with clinical signs of stiffness and a shifting lameness in one or more limbs. However, a clinical diagnosis is often not possible during the early stages of the disease when clinical signs are inconspicuous (Bevan 1907; McFarlane and Haig 1955; Davies et al. 1975; St George et al. 1984). In dairy cattle, the only clinical sign observed may be a drastic drop in milk production (Henning 1956; Theodoridis et al. 1973; Davis et al. 1984). Because of the short duration of the febrile reaction (often less than six hours), the fever associated with infection may easily be missed and

the condition may appear to be an intoxication or nutritional disturbance.

Affected animals reveal certain haematological and biochemical changes which can support a diagnosis of ephemeral fever. The febrile reaction coincides with a pronounced neutrophilia with up to 30% band forms (St George et al. 1984; Uren and Murphy 1985; Young and Spradbrow 1990). Blood collected during the later stages of illness may show poor clot formation (St George, in press). Reduction in blood levels of ionised and bound fractions of calcium also occurs, particularly in those animals manifesting severe signs (St George et al. 1984; Murphy et al. 1986; St George, in press). A response to treatment with calcium borogluconate may thus give supportive evidence for a diagnosis of ephemeral fever.

Studies by Mackerras et al. (1940) showed that infectivity was associated exclusively with the leukocyte-platelet fraction of blood. Subsequently it has been reported that neutrophils and mesothelial cells contain viral antigen (Young & Spradbrow, 1985). Viraemia is usually present for about 4–5 days, commencing one day before the temperature reaction and terminating 1–2 days after clinical recovery (MacFarlane & Haig 1955; St George, in press).

The cumbersome and expensive system of passaging the virus in susceptible cattle was used for many years (MacKerras et al. 1940) until it was demonstrated that BEF virus can be isolated from bovine leucocyte suspensions by intracerebral inoculation of suckling mice (Van der Westhuizen 1967). This technique was responsible for a number of isolations of BEF virus in various countries (Doherty et al. 1979; Spradbrow & Francis 1969; Davies & Walker 1974). The suckling mouse method has now been replaced by cell culture techniques. Cybinski & Muller (1990) demonstrated that rhabdoviruses such as BEF virus and Tibrogargan virus could be isolated from insects or from cattle blood by using C6/36 *Aedes albopictus* cell cultures. However, because of the brief viraemia associated with BEF virus infection, it is not always possible to isolate the virus from field cases. Cell culture isolates can be characterised as members of the BEF virus group by an indirect fluorescent antibody technique (Cybinski & Zakrzewski 1983; Zakrzewski & Cybinski 1984). To specifically identify the isolate, a two-way cross neutralisation test must be used (Doherty et al. 1969; St George 1985).

After primary infection of cattle with BEF virus, an antibody response is first detected 2–3 days after recovery (St George 1980; Young and Spradbrow 1990). Neutralising antibody titres reach their maximum levels 4-5 weeks after infection and high titres of antibody are still present 422 days after

inoculation of the virus (Snowdon 1970). Cattle which fail to react clinically also develop neutralising antibody (Snowdon 1970; Young and Spradbrow 1990). In Australia the serological diagnosis of ephemeral fever is complicated by the existence of a number of antigenically related viruses in the BEFV-group. These viruses may play a role in sensitising cattle, so that an accelerated antibody response follows infection with BEF virus (Cybinski & Zakrzewski 1983; St George et al. 1984). The role of these or similar viruses in southern Africa has not yet been investigated.

Differential Diagnosis

The clinical signs of ephemeral fever are not pathognomonic and could easily be confused with other diseases, for example, post-parturient hypocalcaemia or milk fever. Hypocalcaemia in cases of ephemeral fever is probably related to clinical signs such as anorexia, reduced movements or stasis of the gastrointestinal tract, muscle tremors or shivering and recumbency (St George et al. 1984; Uren and Murphy 1985). Hypocalcaemia may also be induced by oxalate containing plants, resulting in signs similar to those of ephemeral fever, and may exacerbate the already hypocalcaemic state in animals suffering from the disease. More severe clinical disease and a higher mortality rate than usual occurred in well-conditioned cattle grazing virtually pure buffel grass (*Cenchrus ciliaris*) pastures with a high oxalate content (T.D. St George, pers. comm.).

A small proportion of animals suffering from ephemeral fever may be recumbent for a week or longer or may show residual ataxia of the hind quarters (Armfield 1915; Rosen 1931; Gray 1938; Mackerras et al. 1940; MacFarlane and Haig 1955; Henning 1956; Basson et al. 1969; Spradbrow and Francis 1969; Snowdon 1970; Hill and Schultz, 1977). These ataxic, paretic or paralytic symptoms could be confused clinically with those caused by botulism, or diplodiosis. The latter disease is a neuromycotoxicosis affecting cattle grazing on harvested maize infected by the fungus *Diplodia maydis* (Kellerman et al. 1988). The clinical signs are very similar to ephemeral fever and include reluctance to move, incoordination, stiff gait, paresis or paralysis, constipation and occasional muscle tremors. Other conditions which may result in limb stiffness include infection with *Clostridium chauvoei*, infection of joints with bacteria and physical injury to the musculoskeletal system.

In Japan, the clinical signs associated with infection with Ibaraki virus have been confused with ephemeral fever. However in Ibaraki disease there is permanent paralysis of the oesophagus due to degeneration of the striated muscles. Erosions on the

muzzle and in the mouth also occur (Inaba 1975). Viruses related to Ibaraki virus are known to occur in Africa, and it is possible that they may cause a similar clinical condition.

Dyspnoea, tachypnoea, and coarse lung sounds sometimes accompanied by subcutaneous emphysema may be evident in a small proportion of ephemeral fever cases (MacFarlane and Haig 1955; Theodoridis and Coetzer 1979). Similar signs may occur in cattle suffering from acute bovine pulmonary oedema and emphysema, also referred to as atypical interstitial pneumonia or fog fever. This condition is associated with grazing of rapidly growing pastures with high levels of L-tryptophan which is converted to 3-methylindole in the rumen. This chemical is pneumotoxic and results in oedema and hyperplasia of alveolar and bronchial epithelium. In contrast, the lung lesions in ephemeral fever are limited to emphysema and bronchiolitis.

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A Blocking ELISA for the Detection of Specific Antibodies to Bovine Ephemeral Fever Virus

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Abstract

A blocking ELISA for detecting antibodies to bovine ephemeral fever (BEF) virus in cattle is described. In this test, the binding capacity of a monoclonal antibody specific for an epitope on antigenic site G1 of the BEF virus glycoprotein is blocked in the presence of positive serum. The sensitivity of the blocking ELISA was compared with the virus neutralisation test using a total of 380 sera from cattle. Of these, 118 were from an area known to be free of bovine ephemeral fever, 181 from cattle either naturally or experimentally infected with BEF virus, 33 sequential serum samples from a sentinel steer from which Berrimah virus had been isolated, 9 from a sentinel cow from which Kimberley virus had been isolated and a panel of 39 sera supplied as a blind trial.

Taken overall, the blocking ELISA results compared favourably with those of the virus neutralisation tests. The monospecificity of the test was demonstrated using hyperimmune mouse ascitic fluid to other BEF serogroup viruses, namely Kimberley and Berrimah viruses, and the results showed no significant cross reaction. The greater simplicity and sensitivity of the blocking ELISA when compared with the virus neutralisation test makes it the preferred test for the diagnosis and monitoring of clinical bovine ephemeral fever.

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Taxonomy, Morphology and Molecular Biology

In 1967 van der Westhuizen described BEF virus as a rhabdovirus based on the bullet shaped morphology typical of that group. Subsequent serological studies suggested a relationship between BEF virus and the lyssaviruses (of which rabies virus is the best known but is not vector borne). Papers in this section provide further information on relationships among the rhabdoviruses and the rationale for a revised classification is presented.

Until recently, classification of viruses was based on morphology, physio-chemical properties and serological relationships. As knowledge of the genomic organisation of viruses becomes available through molecular biology studies, it is likely that this information will form the basis of future classification. Recent studies on the molecular biology of BEF and related viruses conclude this section.

Taxonomy of Viruses of the Family Rhabdoviridae

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Abstract

The International Committee on Taxonomy of Viruses recently endorsed a recommendation to introduce the taxonomic category 'order' into virus classification. The first families of viruses so placed are in the order Mononegavirales (families Rhabdoviridae, Filoviridae and Paramyxoviridae) which include viruses such as Newcastle disease, mumps, respiratory syncytial, measles and Ebola viruses as well as rabies, bovine ephemeral fever and vesicular stomatitis viruses. Of 92 non-plant rhabdoviruses infecting vertebrates and invertabrates, 20 have been placed in the genus *Vesiculovirus*, 26 in the genus *Lyssavirus* and 46 have not been placed in either genus. This paper describes the molecular, antigenic and biological characteristics of viruses of the family Rhabdoviridae with emphasis on viruses of the genus *Lyssavirus* related to bovine ephemeral fever virus.

THE order Mononegavirales embraces the three families of eukaryotic viruses possessing linear nonsegmented negative-strand RNA genomes. Family Filoviridae includes Ebola and Marburg viruses, family Paramyxoviridae includes Newcastle disease, mumps, respiratory syncytial, pneumonia virus of mice, and measles viruses and family Rhabdoviridae includes rabies, bovine ephemeral fever, and vesicular stomatitis viruses. The family Rhabdoviridae includes two genera, *Lyssavirus* (rabies and bovine ephemeral fever viruses) and *Vesiculovirus* (vesicular stomatitis viruses).

The name Mononegavirales is from the Greek word monos, meaning 'single'; nega from negative-strand RNA; and virales meaning 'viruses'. Common features of these families include a negative-sense template RNA in the virion, a helical nucleocapsid, initiation of primary transcription by a virion-associated RNA-dependent RNA polymerase, similar gene order (3'-core protein genes, envelope protein genes, polymerase gene-5'), and a single 3' promoter. The viruses mature by budding, principally from the plasma membrane, or less frequently from internal membranes (rabies virus) or the inner nuclear membrane (many plant rhabdoviruses). Replication is cytoplasmic, except for some plant rhabdoviruses.

Members of the three families which comprise the Mononegavirales can be distinguisehed by virion morphology. The Filoviridae are characterised by simple, branched, U-shaped or 6-shaped filaments which are circular in cross section and of uniform diameter (approximately 80 nm) but which may extend up to 14 000 nm in length. Purified virions are bacilliform and of uniform length. Members of the Paramyxoviridae are characterised by filamentous, pleomorphic or spherical structures of variable diameter while the Rhabdoviridae are characterised by regular bullet-shaped or bacilliform particles. The helical ribonucleoprotein core of Mononegavirales has a diameter of 13–20 nm, which in filoviruses and rhabdoviruses is organised into a helical nucleocapsid, approximately 50 nm in diameter.

While non-plant rhabdoviruses are bullet-shaped, those infecting plants usually are bacilliform. The virions of both plant and animal rhabdoviruses are 100–430 nm long and 45–100 nm in diameter, with surface projections (G protein) 5–10 nm long and about 3 nm in diameter (Wunner et al. 1991). In thin sections, a central axial channel is seen. Characteristic cross-striations with 4.5–5.0 nm spacings are seen in negatively stained and thin-sectioned particles. Truncated particles may be common, except perhaps in members infecting plants. Abnormally long and double-length particles

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and tandem formations are sometimes observed. A honeycomb pattern appears on the surface of some members. The inner nucleocapsid is approximately 50 nm in diameter with helical symmetry. It consists of an RNA + N protein complex together with L and NS proteins, surrounded by an envelope containing M protein. The nucleocapsid contains transcriptase activity and is infectious. It uncoils to a helical structure 20×700 nm.

Five major polypeptides have been identified and designated L, G, N, NS, and M for vesicular stomatitis virus and L, G, N, M1, and M2 for BEF virus (Walker et al. 1991). The G protein is involved in virus neutralisation and defines the serotype. N protein shows cross-reactions between some vesiculoviruses and between some lyssaviruses.

Rhabdoviruses have a great variety of shape, size, hosts, mechanisms of transmission, vectors, disease, geographic distribution, and importance. Some members replicate in arthropods as well as in vertebrates, others in arthropods and plants. Some that replicate in vertebrates have a wide experimental host range. Some are transmitted vertically in insect vectors, including mosquitoes, sandflies, culicoides, mites, aphids, or leafhoppers. Only rabies virus has been shown to be vertically transmitted in vertebrates (Steece and Calisher 1989), but none of the plant rhabdoviruses have been shown to be vertically transmitted.

Until recently, new rhabdovirus isolates were classified principally, but not solely, on the basis of morphology and their antigenic relationships with recognised rhabdoviruses. The use of antigenic site mapping of viral glycoproteins by monoclonal antibody analyses has complemented, and to some degree replaced, more classical methods (Cybinski et al. 1990). Molecular virology, in particular molecular epidemiology, has provided additional information for genetic taxonomic placements. It is clear that many rhabdoviruses, including BEF virus, do not fall neatly into either of the two established genera *Lyssavirus* or *Vesiculovirus* and sequence homology data may result in the establishment of new genera.

Meanwhile, of 195 rhabdoviruses and rhabdovirus-like viruses currently recognised by the International Committee on Taxonomy of Viruses (Wunner et al. 1991; Calisher and Fauquet, in press), 90 are plant rhabdoviruses (Table 1). The remaining 105 viruses have been isolated from vertebrates or invertebrates. Table 2 lists the lyssaviruses, vesiculoviruses, probable rhabdoviruses assigned to serogroups, and probable rhabdoviruses not assigned to serogroups.

None of the plant rhabdoviruses are known to be antigenically related to any of the animal or insect

Table 1. Plant rhabdoviruses.

Plant rhabdovirus subgroup A: Barley yellow striate mosaic, Broccoli necrotic yellows, *Datura* yellow vein, *Festuca* leaf streak, Lettuce necrotic yellows, Maize mosaic, Northern cereal mosaic, *Sonchus*, Strawberry crinkle, Wheat American striate mosaic

Plant rhabdovirus subgroup B: Potato yellow dwarf, Eggplant mottled dwarf, *Sonchus* yellow net, Sowthistle yellow vein

Probable rhabdoviruses: Beet leaf curl, Carrot latent, Cereal chlorotic mottle, *Chrysanthemum frutescens*, Coffee ring spot, *Colocasia* bobone disease, Coriander feathery red vein, Cow parsnip mosaic, *Cynara*, *Digitaria* striate, Finger millet mosaic, *Gomphrena*, Lucerne enation, Maize sterile stunt, Oat striate mosaic, Papaya apical necrosis, Parsley latent, *Pelargonium* vein clearing, *Pisum*, *Pittosporum* vein yellowing, Raspberry vein chlorosis, Rice transitory yellowing, Sorghum stunt, Sorghum stunt mosaic, Wheat chlorotic streak, Wheat rosette stunt, Winter wheat Russian mosaic, *Raphanus*

Possible rhabdoviruses: *Atropa belladonna*, *Callistephus chinensis* chlorosis, Caper vein yellowing, Carnation bacilliform, Cassava symptomless, *Chondrilla* stunting, *Chrysanthemum* vein chlorosis, Clover enation, *Cynodon* chlorotic streak, Endive, *Euonymus* fasciation, *Gerbera* symptomless, *Gloriosa* fleck, *Holcus lanatus* yellowing, Honeysuckle vein chlorosis, *Iris germanica* leaf stripe, Ivy vein clearing, *Laburnum* yellow vein, *Laelia* red leaf spot, *Launea arborescens* stunt, Lemon scented thyme leaf chlorosis, *Lolium* (ryegrass), *Lotus* streak, Lupin yellow vein, *Malva silvestris*, *Melilotus* latent, Melon leaf variegation, *Mentha piperita* latent, Passionfruit vein clearing, Patchouli (*Pogostemon patchouli*) mottle, Peanut veinal chlorosis, Pigeon pea (*Cajanus cajan*) proliferation, Pineapple chlorotic leaf streak, Plantain (*Plantago lanceolata*) mottle, *Ranunculus repens* symptomless, Red clover vein mosaic, *Saintpaulia* leaf necrosis, *Sambucus* vein clearing, Sarracenia purpurea, Strawberry latent C, Tomato vein clearing, *Triticum aestivum* chlorotic spot, *Vigna sinensis* mosaic, *Zea mays*

Nonenveloped rhabdovirus-like particles: *Citrus* leprosis, Orchid fleck, *Dendrobium* leaf streak, *Phalaenopsis* chlorotic spot

rhabdoviruses. Plant rhabdoviruses are included in the family on the basis of shape, size, nucleic acid and other characters and properties. Nevertheless, molecular studies of all these viruses provide evidence for distant evolutionary commonalities and further evidence confirming their relationships is to be expected.

Thirty-two viruses belong to the genus *Lyssavirus*, which includes both bovine ephemeral fever (BEF) and rabies viruses, as well as an antigenic and, perhaps, genetic continuum of viruses in between. Twenty-nine viruses belong to the genus *Vesiculovirus*, which includes the viruses causing vesicular stomatitis. Forty-four other viruses have sufficient characteristics in common with recognised

Table 2. Viruses placed in the genera *Lyssavirus* or *Vesiculovirus* or which have not been placed in a genus.

Lyssaviruses	Adelaide River, Berrimah, Bivens Arm, **bovine ephemeral fever**, Charleville, Coastal Plains, Duvenhage, eel B12, European bat 1, European bat 2, Hirame rhabdovirus, Humpty Doo, Infectious haematopoietic necrosis, Kimberley, Kolongo, Kotonkan, Lagos bat, Malakal, Mokola, Nasoule, Ngaingan, Oak-Vale, Obodhiang, Parry Creek, Puchong, Rabies, Rochambeau, Sandjimba, Snakehead rhabdovirus, Sweetwater Branch, Tibrogargan, viral haemorrhagic septicemia
Vesiculoviruses	BeAn 157575, Boteke, Calchaqui, Carajas, Chandipura, Cocal, Eel American/Eel European, grasscarp rhabdovirus, Gray Lodge, Isfahan, Jurona, Klamath, Kwatta, La Joya, Malpais Spring, Maraba, Mount Elgon bat, Perinet, Pike fry rhabdovirus, Piry, Porton, Radi, Spring viremia of carp, Tupaia, ulcerative disease rhabdovirus, vesicular stomatitis Alagoas, vesicular stomatitis Indiana, vesicular stomatitis New Jersey, Yug Bogdanovac
Probable members of family Rhabdoviridae	**Bahia Grande serogroup** (Bahia Grande, Muir Springs, Reed Ranch), **Hart Park serogroup** (Flanders, Hart Park, Kamese, Mosqueiro, Mossuril), **Kern Canyon serogroup** (Barur, Fukuoka, Kern Canyon, Nkolbisson), **Le Dantec serogroup** (Keuraliba, Le Dantec), **Sawgrass serogroup** (Connecticut, New Minto, Sawgrass), **Timbo serogroup** (Chaco, Sena Madureira, Timbo)
No serogroup assigned	Almpiwar, Aruac, Atlantic cod ulcus syndrome, Bangoran, Bimbo, DakArK 7292, Gossas, Joinjakaka, Kannamangalam, Landjia, Marco, Mn936-77, Navarro, Oita 296, Ouango, perch rhabdovirus, rhabdovirus of blue crab, rhabdovirus of entamoeba, Rhabdovirus salmonis, Rio Grande cichlid, Sigma, Sripur, Xiburema, Yata.

rhabdoviruses to be considered probable members of the family. In summary, BEF virus is a member of the genus *Lyssavirus*, family Rhabdoviridae, order Mononegavirales.

A good understanding of taxonomy leads to better laboratory diagnosis and to better treatment. A better understanding of the genetic and phenetic relations between BEF virus and its close relatives (Kimberley, Berrimah, Adelaide River, Malakal, and Puchong viruses) will allow us to better understand the evolution and epidemiology of these viruses. As an example, Adelaide River virus is a close antigenic relative of BEF virus but is detected by monoclonal antibody to Rabies virus. Because of cross-reactivity between Adelaide River and BEF viruses, even a rise in titre of antibody to BEF virus does not necessarily indicate recent BEF virus infection (Cybinski, 1987). It is of epidemiologic importance to note that subclinical infections with related viruses may limit an ephemeral fever epidemic by providing temporary protection by interference (St George, 1985).

Thus, identification of viruses should be precise and accurate. Giving viruses names when none is needed or warranted, or not giving them names when such is warranted, and using poor descriptives are disservices to the scientific community and to journal editors. If BEF-like viruses that do not cause disease are all given the name BEF virus, a horrid situation, such as we have with the taxonomy of bluetongue viruses, will result.

Finally, development of efficacious vaccines must depend not only on an understanding of the underlying pathophysiology of the disease but on specific determination of the etiologic agent causing that disease. An internationally accepted taxonomy and hierarchy are the only reasonable bases on which to report such viruses.

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Antibody to Bivens Arm Virus in Trinidadian Buffalo: A Temporal and Evolutionary Link between Australia, Asia and the Caribbean?

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Abstract

In 1981, Bivens Arm virus (family Rhabdoviridae, genus *Lyssavirus*) was isolated from *Culicoides insignis* collected near water buffalo (*Bubalus bubalis*) in Gainesville, Florida, USA. This virus is closely related to Sweetwater Branch virus, (which was also isolated from *C. insignis* in Florida) and to Tibrogargan virus (a distant relative of bovine ephemeral fever virus) isolated from *C. brevitarsis* in Australia. When stored sera were tested for antibody to Bivens Arm virus, the results indicated that the virus was present in Florida prior to the 1981 arrival of water buffalo. Because the water buffalo had originated in Trinidad, 315 serum samples were collected from Trinidadian water buffalo at three sites and tested for neutralising antibody to Bivens Arm, Sweetwater Branch, and Tibrogargan viruses, and to the vesiculoviruses, vesicular stomatitis New Jersey, vesicular stomatitis Indiana, and Cocal viruses.

Overall prevalence rates for the vesiculoviruses were: 53 (17%) with antibody only to vesicular stomatitis New Jersey virus, 24 (8%) with antibody only to vesicular stomatitis Indiana virus and 14 (4.4%) with antibody only to Cocal virus. Of the remaining 224 buffalo, 23 (7%) had antibody to more than one of these vesiculoviruses. Antibodies to Bivens Arm, Sweetwater Branch, and Tibrogargan viruses were detected in 64 (20%) animals. Of these, 30 (10%) had antibody only to Bivens Arm virus, 20 (6%) to Bivens Arm, Sweetwater Branch, and Tibrogargan viruses, 11 (4%) to Bivens Arm and Tibrogargan viruses, 2 (0.6%) only to Sweetwater Branch virus, and 1 (0.3%) to Bivens Arm and Sweetwater Branch viruses; none had antibody to Tibrogargan virus alone. Antibody was not evenly distributed among the three groups; buffalo at locations in the north and the east of the island had antibody to these viruses while those at a site in the far south-western tip of Trinidad did not. We interpret our results to indicate that Tibrogargan virus does not occur in Trinidad. However, it appears that Bivens Arm virus, or a close relative of it, does occur in Trinidad.

In 1981, Bivens Arm virus (family Rhabdoviridae, genus *Lyssavirus*) was isolated from *Culicoides insignis* collected near water buffalo (*Bubalus bubalis*) in Gainesville, Florida; the water buffalo had been born in Trinidad. This virus is closely related to Sweetwater Branch virus, also isolated from Florida *C. insignis*, and to Tibrogargan virus, isolated from *C. brevitarsis* in Australia (Gibbs et al. 1989). Testing of stored serum samples from domestic cattle in Florida indicated that infection with Sweetwater Branch virus predated the 1981 arrival of water buffalo in Florida. To determine whether Trinidadian water buffalo also have antibody to Bivens Arm virus and to determine the prevalence of antibody to Sweetwater Branch and Tibrogargan viruses as, 315 serum samples were collected in 1990 from water buffalo at three sites in Trinidad and tested for neutralising antibody to Bivens Arm, Sweetwater Branch, and Tibrogargan viruses. These samples were also tested for antibody to the vesiculoviruses, vesicular stomatitis New Jersey, vesicular stomatitis Indiana and Cocal

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viruses. The results suggest that Bivens Arm virus, or a closely related virus, occurs in Trinidad, but Tibrogargan and Sweetwater Branch viruses do not.

Materials and Methods

Serum collections and neutralisation tests

The island of Trinidad is situated off the north-eastern coast of Venezuela between latitudes $10°3'$ and $10°50'$ N and longitudes $60°55'$ and $61°56'$ W. Serum samples were collected in 1988 specifically for this study from part of a large consignment of buffalo assembled for export to Central America. The animals were obtained from three different parts of Trinidad; Malabar (St George County) in the north-west, Cocal (Nariva/Mayaro County) in the east, and Icacus (St Patrick County) in the south-west. Serum samples were heat inactivated (56 °C for 30 minutes) and frozen for later testing. Ages of the animals were not available, but those from the south-western location were younger than those from the other two sites. Serum samples were sent by air, under United States Department of Agriculture licence, to the University of Florida, Gainesville, then transhipped to the Center for Disease Control in Fort Collins. Plaque reduction neutralisation tests were used to detect the presence of antibody to specific viruses (Lindsey et al. 1976). Briefly, 100 plaque forming units of prototype viruses (Bivens Arm, strain BT82-55; Sweetwater Branch, strain UF-11; and Tibrogargan, strain CSIRO-132) were used in a microtitre Vero cell culture assay. The diluent for serum samples and for viruses was Medium 199 with 1% bovine albumen, pH 7.4. To identify those sera with antibody, samples were initially screened at 1:20 final dilutions (serum diluted 1:10 mixed with an equal volume of 200 plaque-forming units of virus). Only Serum samples that contained antibody to Tibrogargan serogroup viruses were further titrated. Samples containing antibody to vesiculoviruses were not tested for end point titres because of the low degree of cross-reactivity among these viruses.

Results

Antibody to lyssaviruses

A total of 64 (20%) water buffalo had neutralising antibody to Bivens Arm, Sweetwater Branch, or Tibrogargan viruses (Table 1). Of these, 30 (10%) had antibody only to Bivens Arm virus, 20 (6%) to Bivens Arm, Sweetwater Branch, and Tibrogargan viruses, 11 (4%) to Bivens Arm and Tibrogargan viruses, 2 (0.6%) only to Sweetwater Branch virus, and 1 (0.3%) to Bivens Arm and Sweetwater Branch viruses; none had antibody to Tibrogargan virus alone.

None of 43 water buffalo at Icacus, 29/133 (22%) at Cocal, and 35/136 (26%) at Malabar had antibody to Bivens Arm, Tibrogargan and Sweetwater Branch viruses. At Cocal, of 29 water buffalo with antibody to one or more of these viruses, 25 had antibody only to Bivens Arm virus; 1 had antibody only to Sweetwater Branch virus; 2 had antibody to Bivens Arm and Tibrogargan viruses; and 1 had antibody to all three viruses. At Malabar, 5/35 with antibody to one or more of these viruses had antibody only to Bivens Arm virus; 12 others had antibody to all three viruses; and 18 had antibody to two of these viruses in different combinations.

Antibody to vesiculoviruses

Of 315 water buffalo, 53 (7%) had antibody to vesicular stomatitis New Jersey but not to vesicular stomatitis Indiana or Cocal virus, 24 (8%) had antibody to vesicular stomatitis Indiana but not to vesicular stomatitis New Jersey or Cocal virus, and 14 (4%) had antibody to Cocal but not to vesicular stomatitis New Jersey or vesicular stomatitis Indiana virus (data not shown). Of the remaining 224 animals, 23 (7%) had antibody to more than one of these vesiculoviruses and 201 (64%) had antibody to none of them. Twelve of 43 (28%) water buffalo at Icacus, 57/133 (43%) at Cocal, and 44/136 (32%) at Malabar had antibody to one or more of these vesiculoviruses. Of 12 water buffalo with antibody at Icacus, 6 had antibody only to vesicular stomatitis New Jersey, 3 only to vesicular stomatitis Indiana; and 1 only to Cocal virus. Of 57 animals at the Cocal site; 24 had antibody only to vesicular stomatitis Indiana; 12 only to vesicular stomatitis New Jersey; 6 only to Cocal; 5 to vesicular stomatitis New Jersey and vesicular stomatitis Indiana; 4 to vesicular stomatitis Indiana and Cocal; 3 to vesicular stomatitis New Jersey and Cocal; and 3 to all three viruses. Finally, at Malabar, of 44 animals with antibody, 26 had antibody to vesicular stomatitis New Jersey only; seven to Cocal only; 7 to vesicular stomatitis Indiana only; 3 to vesicular stomatitis New Jersey and vesicular stomatitis Indiana; 1 to vesicular stomatitis Indiana and Cocal; and 1 to vesicular stomatitis New Jersey and Cocal viruses.

Discussion

A previous serological survey of two small groups of cattle raised in St. Croix and Puerto Rico, in the north-eastern Caribbean, established that antibody to Bivens Arm virus, or a virus closely related to it, exists on both islands (Tuekam et al. 1991). Our results indicate that Bivens Arm virus, or a virus closely related to it, also occurs in Trinidad and infects water buffalo. Antibody was detected in water buffalo in the north and the east of Trinidad but not

Table 1. Serum dilution-plaque reduction neutralising antibody titres of water buffalo with antibody to the lyssaviruses Bivens Arm (BA), Sweetwater Branch (SB) and Tibrogargan (TIB).

Serum no.	Titre of antibody to:			Serum no.	Titre of antibody to:		
	BA	SB	TIB		BA	SB	TIB
2	320	20	80	171	80	—	—
19	10	40	160	174	40	10	—
20	10	20	80	176	20	—	20
32	—	10	—	180	320	—	—
34	20	—	10	184	20	—	—
35	320	320	320	205	20	—	—
36	20	20	10	208	40	—	—
40	80	—	40	213	20	—	—
45	160	—	—	218	20	—	—
46	320	40	80	219	20	—	—
56	80	10	10	226	160	—	20
67	160	—	40	228	20	—	—
69	20	10	20	234	20	—	—
70	80	—	20	241	20	—	—
74	320	320	320	242	20	—	—
84	80	40	40	243	40	—	—
94	80	—	320	246	40	—	—
142	320	20	20	249	20	—	—
143	40	—	—	251	40	—	—
146	160	—	40	253	40	—	—
147	160	10	20	263	160	—	—
148	20	80	40	264	160	80	160
149	20	80	40	269	10	—	—
154	80	80	80	270	80	—	—
155	20	—	160	277	40	—	—
156	80	40	160	280	20	—	—
161	20	20	20	281	20	—	—
162	160	—	20	284	40	—	—
163	160	—	—	285	10	—	—
164	320	320	320	286	10	—	—
165	160	80	320	287	20	—	—
170	20	—	10	315	—	20	—

— signifies < 10

at a site in the far southwestern tip. We did not obtain evidence for the presence of Tibrogargan virus in Trinidad.

Bivens Arm virus is a member of the Tibrogargan serogroup which is comprised of viruses isolated in Australia and Florida. The Australian viruses are Tibrogargan virus, isolated from *C. brevitarsis*; Coastal Plains virus, isolated from the blood of a healthy *Bos taurus* steer, Ngaingan virus isolated from *C. brevitarsis*, and, perhaps, others as described by Calisher et al. (1989). The viruses in the group isolated in Florida are Bivens Arm and Sweetwater Branch viruses. It is possible that other Tibrogargan serogroup viruses occur in other parts of the world and that they are transmitted by *Culicoides* spp. midges. However, except for those mentioned, none have been isolated, probably because there is little interest in arboviruses that apparently are non-pathogenic. This is short-sighted because these and other rhabdoviruses, such as Tibrogargan virus have been shown to be related to both bovine ephemeral fever and rabies viruses. Cattle injected with Tibrogargan and other lyssaviruses (including viruses clearly unrelated to bovine ephemeral fever virus) appear to be temporarily protected against development of clinical ephemeral fever (St George 1985). Although such protection may only be transient, any relationship between Tibrogargan, bovine ephemeral fever, and rabies viruses suggests the possibility not only of diagnostic confusion but natural protection as well.

Given the expected, but as yet unproven, transmission of Bivens Arm virus by *Culicoides* spp. midges in Florida, it seems likely that virus isolation

attempts from these biting flies in Trinidad would be productive in terms of isolating the virus infecting the Trinidadian water buffalo. Cybinski and Gard (1986) suggested that because the geographical distribution of neutralising antibody to Coastal Plains virus in Australian cattle corresponded to the distribution of *C. brevitarsis*, this virus may be arthropod-borne and transmitted by this species.

In the 1970s, several consignments of water buffalo were brought from Australia to Venezuela and transhipped through the airport in Trinidad. It is conceivable, but unproven, that some were off-loaded in Trinidad. In the 1980s, several shiploads of water buffalo were also imported into Cuba. It is tempting to speculate, but difficult to prove, that infection with lyssaviruses in Trinidad may be due to the international trade of water buffalo.

The detection of antibodies to the vesiculoviruses, vesicular stomatitis New Jersey, vesicular stomatitis Indiana, and Cocal viruses in Trinidad is not surprising in view of the fact that vesicular stomatitis New Jersey and vesicular stomatitis Indiana viruses are endemic in Venezuela (Webb and Holbrook 1989) and Cocal virus was first isolated in Trinidad (Jonkers et al. 1964). The circumstances of isolation, seroepidemiology, ecology, and characteristics of arboviruses of the Caribbean, including Trinidad, have been reviewed in a remarkable paper by Spence et al. (1968). The occurrence of serotype specific antibody to vesicular stomatitis New Jersey (17%), vesicular stomatitis Indiana (8%), and Cocal (4%) in our study suggests that all three viruses occur in Trinidad; virus isolation studies are required to determine whether this is correct. Of further interest was the fact that only 7% of the other animals tested had antibody to more than one of these vesiculoviruses. On the basis of the occurrence of higher prevalence rates to vesicular stomatitis New Jersey and vesicular stomatitis Indiana virus and 4% to Cocal virus, one would expect some additive effect, such that antibody to two or three of these viruses would total more than the 16% found for vesicular stomatitis

New Jersey. That the total is less (7%) supports previous observations (reviewed by Webb and Holbrook, 1989) that little cross— reaction (and cross-protection) occurs between these viruses. The data suggest that in Trinidad these vesiculoviruses exist in distinct ecosystems.

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Studies on the Morphology of a Chinese Isolate of Bovine Ephemeral Fever Virus

Li Cheng*, Gu Shoulin*, Jiang Shaode*, Hao Guiyu* and Zhang Tan*

Abstract

Bovine ephemeral fever virus (Beijing 1) particles were observed to be bullet or cone shaped, with an average length of 180 nm and diameter of 85 nm. After BHK21 cell culture passage, there was an increase in the number of truncated particles observed. Mature particles were released by budding from cytoplasmic vesicles and endoplasmic reticulum of the host cell. The complete virus particle consists of spikes, a membranous envelope and a helical nucleocapsid. The spikes were easily disrupted and were often not seen on viruses purified by physico-chemical procedures. Inclusions were usually found within the cytoplasm of infected cells, and consisted of virions of different shapes as well as matrices and membranous material, and were occasionally surrounded by intermembranous structures.

BOVINE ephemeral fever (BEF) virus was first isolated in mice by van der Westhuizen (1967) and the virus was subsequently adapted to grow in BHK21 cell cultures. The morphology of the virus has been described by workers in South Africa (Lecatsas 1969), Japan (Ito et al. 1969) and Australia (Tzipori 1975). BEF virus is a member of the genus *Lyssavirus*, family Rhabdoviridae, Order Mononegavirales (see Callisher, these proceedings).

In this paper, we report on the morphology of the Chinese isolate known as Beijing 1 (see Liu Shanggao, these proceedings). The structure, size, replication and distribution of virus in infected cells was determined. Observations were also made on the relationship between viral morphology and virulence.

Materials and Methods

BHK21 cell cultures, infected with BEF virus (Beijing 1) were fixed with glutaraldehyde and osmium tetroxide, dehydrated in acetone and embedded in epoxy resin then sectioned with an LKB microtome. Sections were stained with uranyl acetate and lead citrate and examined with JEM-6C and JEM-1200 EX transmission electron microscopes.

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For negative staining, virus obtained from cell culture fluids was prepared by differential and gradient centrifugation. A drop of the concentrated virus solution was placed onto a carbon–formvar grid and the excess solution absorbed by filter paper. Samples were then stained with 2% phosphotungstic acid.

For scanning electron microscopy, cell cultures were grown on glass slides and infected with virus. The monolayers were fixed with glutaraldehyde and osmium tetroxide, dehydrated in ethanol, treated with valerate and then dried in a dessicator before examination with a JSM-35C scanning electron microscope.

Results

Using negative staining techniques, BEF virus appeared variously as bullet, cone or globe shaped particles, or as conical particles with narrow or broad bases. Sizes were estimated to be 180 x 85 nm. Virions consisted of envelopes (including spikes and viral membranes) and nucleocapsids. Whole virus particles showed a hollow appearance. Viral membranes appeared as two-layer unit membranes with a thickness of about 10 nm (Figure 1). Nucleocapsids were helical with a diameter of about 15 nm and a pitch of 5 nm and had a helical appearance. In cell cultures inoculated with attenuated BEF viruses,
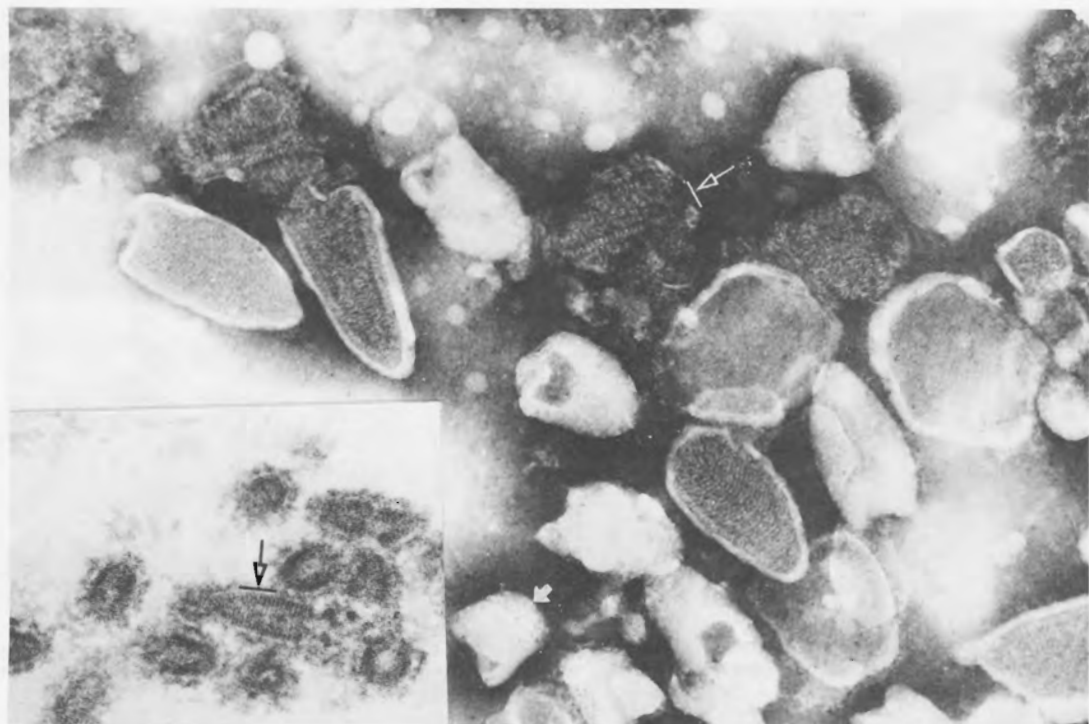
Fig. 1. Negatively-stained BEF virus showing bullet and cone-shaped virus particles, as well as truncated particles and disrupted helical nucleocapsids (arrow) (x 150 000). The insert shows virus particles in thin sections, striations can be clearly seen (arrow) (x 75 000).

there was an increase in the number of cylindral or spherical particles.

BEF virus assembly was observed to occur in the cytoplasm of host cells. Mature viruses were released into vesicles or spaces within host cells by a process of budding. The shapes of the buds were mainly conical or bullet-like, although various types were seen in sections (Figures 2, 3, 4, 5).

In the cytoplasm of infected cells, inclusions were formed by the coalescence of virus particles which tended to force other cell organelles aside. In addition to virions of various shapes, there were also uniform virus matrices, disrupted virions and membranous materials. Boundary membranes were occasionally seen around inclusions.

Examination of infected cell cultures by scanning electron microscopy revealed the presence of spherical, semi-spherical, cylindral or conical projections on the surfaces of infected cells. These features represented immature virions budding from the cell surface and mature virions released on the cell surface.

Discussion

Using transmission and scanning electron microscopy we found that BEF virus which had been propagated in BHK21 cell cultures was commonly bullet or cone-shaped, 180 x 85 nm in size and located within cytoplasmic vesicles, budding from membranes or as inclusions. These observations were similar to previous reports (Ito et al. 1969; Murphy et al. 1972; Theodoridis and Lecatsas 1973; Tzipori 1975).

Many round and short-cylindrical virus particles, which had the appearance of truncated, defective interfering particles, were found in sections of cell cultures which had been infected with attenuated virus. These truncated particles have been reported to be non-infective and to produce auto-interference (that is, their replication reduces normal virus replication, resulting in a decrease in virus titre). In our observations, the number of truncated particles appeared to increase with virus passage.

On the surfaces of infected cells, spherical, semi-spherical and bullet-shaped projections were observed by scanning electron microscopy. These

85

Fig. 2. Various budding viruses (arrow) were observed on the membranes of cytoplasmic vesicles in thin sections of infected cells (x 35 000).

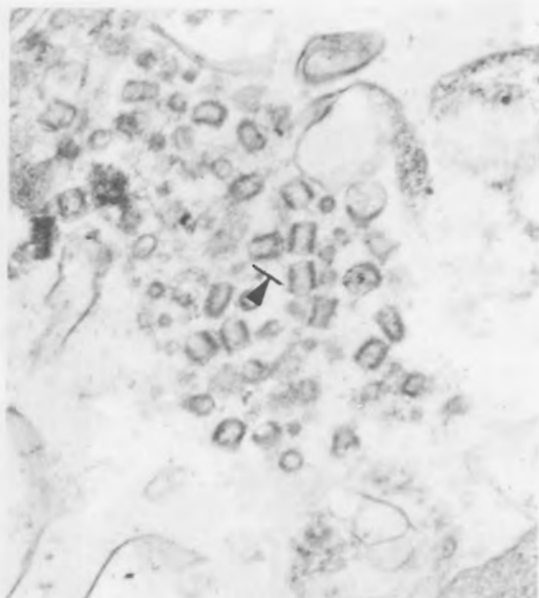**Fig. 3.** Short cyclindrical virus particles (arrow) were observed in thin sections of cells infected with cell culture attenuated virus (x 35 000).
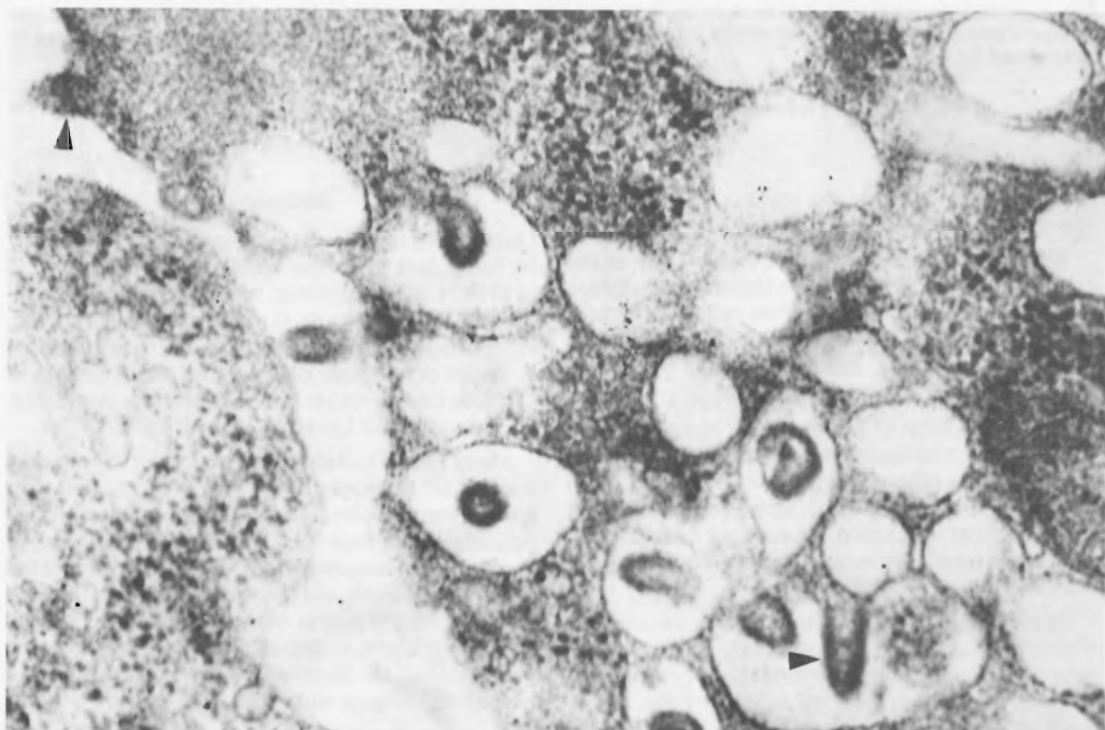

Fig. 4. Bullet-shaped budding viruses (arrows) in thin sections of infected cells (x 50 000).
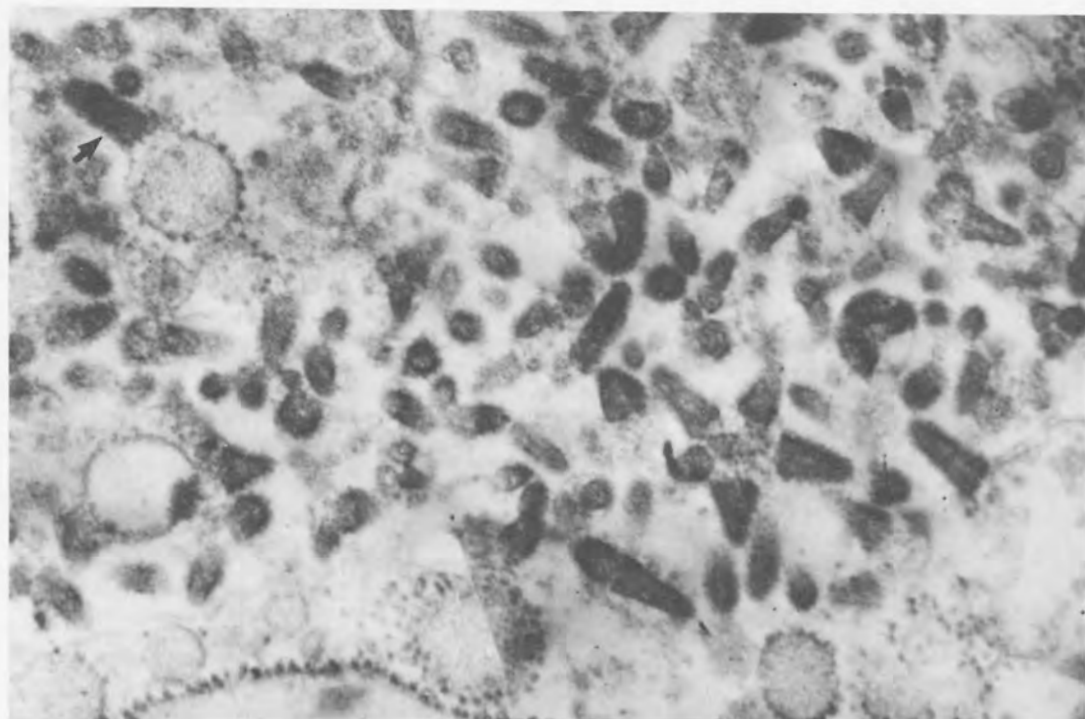
Fig. 5. Thin section of infected BHK21 cells showing bullet, truncated, round and cylindrical particles (x 50 000).

projections were considered to be BEF virus particles because they were present only in the inoculated samples, and not in uninoculated controls. Similar size particles were also observed by transmission electron microscopy. Our observations confirm that a Chinese isolate of BEF virus has typical rhabdovirus morphology.

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The Molecular Biology of Bovine Ephemeral Fever Virus

P.J. Walker*

Abstract

Bovine ephemeral fever virus is an arthropod-borne rhabdovirus which has bullet-shaped morphology and contains a negative sense RNA genome and at least 5 structural proteins (L, G, N, M1 and M2) which appear to correspond to those of rabies virus and vasicular stomatitis virus. The detection of direct and indirect serological cross-reactions between BEF virus, Adelaide River virus and rabies virus has recently resulted in the classification of BEF virus and a number of other insect-transmitted rhabdoviruses in the genus *Lyssavirus*. However, in host range and mode of transmission, BEF virus resembles viruses of the *Vesiculovirus* genus.

The nucleotide sequence of the genome of BEF virus and most of the genome of Adelaide River virus has been determined. The BEF virus genome is a 14.8 kb RNA which contains nine identified genes in the order 3'-N-M1-M2-G-GNS-α-β-γ-L-5'. The genome is 25–30% larger than the genomes vesicular stomatitis virus and rabies virus, primarily due to the presence of a 3.4 kb region between the G and L genes which contains a second glycoprotein gene (GNS) and three genes encoding putative non-virion proteins α, β, γ. The GNS gene encodes a 90 kD non-structural glycoprotein that shares significant amino acid sequence homology with the BEF virus G protein and the G proteins of other rhabdoviruses. The region downstream of the GNS gene has a complex organisation and appears to encode novel proteins. The α and β genes each contain 2 consecutive open reading frames (α_1 and α_2; β_1 and β_2) interrupted by stop codons. The γ gene contains a single open reading frame encodes a 13.5 kD polypeptide. Adelaide River virus has a similar organisation but lacks the γ gene. The structural organisation and amino acid sequence relationships of each of these viruses suggest that they should constitute the first members of a new genus of rhabdovirus to be called *Ephemerovirus*.

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Genome Organisation and Amino Acid Sequence Relationships of Adelaide River Virus

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Abstract

Adelaide River virus was isolated from a healthy sentinel steer near Darwin in 1981 and was shown to cross-react by indirect immunofluorescence with bovine ephemeral fever virus and the closely related rhabdovirus Kimberley. More recent serological studies have shown that Adelaide River virus is also related by indirect immunofluorescence to rabies virus, Duvenhague virus and the rabies-related virus Obodhiang. The cross-reactions appear to be at least partly due to the nucleoprotein (N protein) which shares epitopes with both bovine ephemeral fever (BEF) virus and rabies virus. On the basis of these cross-reactions, Adelaide River and BEF viruses have been classified in the genus *Lyssavirus*. We have determined the genome organisation of Adelaide River virus by nucleotide sequence analysis of cDNA clones obtained from genomic RNA. The 14.6 kb (-) RNA genome contained eight genes which were arranged in the order 3 '-N-M1-M2-G-GNS-α-β-L-5 '. Each gene shared highly significant amino acid sequence homology with the corresponding BEF virus gene product. Significantly, Adelaide River virus contained a complex organisation in the G-L region, including two glycoprotein genes, but lacked a gene corresponding to the BEF virus γ gene. Each gene was flanked by putative consensus and polyadenylation sequences. The α gene contained two consecutive open reading frames (α_1 and α_2) interrupted by stop codons. An analysis of amino acid sequence relationships of the nucleoproteins (N proteins) indicated that Adelaide River virus is most closely related to the BEF virus and that both viruses are more closely related to vesicular stomatitis virus serotypes that to rabies virus. The serological reactions with rabies virus may be explained by short regions of highly conserved sequence that do not reflect the overall relationships of the proteins.

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In Vitro Cleavage of Bovine Ephemeral Fever Virus mRNA Species with Synthetic Hammerhead Ribozymes

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Abstract

Ribozymes are RNA molecules with secondary structures that afford enzymatic, RNA-cleaving activities. By inserting the catalytic domain of a hammerhead ribozyme into RNA sequences complementary to any unique RNA sequence, ribozymes can be designed to associate specifically with a target via base-pairing, cleave the RNA substrate and subsequently dissociate to catalyse further cleavage events. We have constructed and targeted synthetic hammerhead ribozymes to the polymerase (L), nucleoprotein (N) and putative non-virion (NV3) mRNAs of bovine ephemeral fever (BEF) virus. BEF virus cDNA and oligonucleotides representing specific ribozyme RNA sequences were cloned into pGEM plasmids downstream of the T7 RNA promoter. Synthetic RNA transcripts of BEF virus RNA species and ribozymes were prepared using T7 RNA polymerase and reacted *in vitro* in the presence of magnesium ions. Specific cleavage was demonstrated with three ribozymes to a clone (m89) of 3'-L-gene mRNA, two to a clone (m85) of 5'-L-gene mRNA, two to a clone (m88) of N-gene mRNA and one to a clone (g238 X-E) of the NV3-gene mRNA. Factors that affect cleavage efficiency, including temperature, substrate RNA secondary structures and reaction conditions are discussed, together with ribozyme cleavage of native BEF virus mRNA *in vitro*, liposome delivery of ribozymes into BEF virus-infected cells and the potential application of ribozymes as therapeutic antiviral agents.

THE term ribozyme has been used to describe RNA molecules with secondary structures that mediate autocatalytic cleavage (Edgington 1992; Sheldon and Symons 1989). For some small circular RNAs of plant viroids and satellite RNAs associated with some plant viruses, the ribozyme catalytic domains have characteristic 'hammerhead-shaped' secondary structures (Buzayan et al. 1986; Forster and Symons 1987. By dissecting the catalytic domain into bi- or trimolecular components, hammerhead ribozymes can be assembled in several configurations to catalyse intermolecular RNA cleavage (Uhlenbeck 1987; Haseloff and Gerlach 1988; Koizumi et al. 1989). Of these *trans*-acting hammerhead ribozymes, the configuration devised by Haseloff and Gerlach (1988) provides the practical advantage that only three nucleotides are required in the substrate RNA to complete the catalytic domain of the ribozyme. The remainder of the ribozyme catalytic domain was inserted into synthetic antisense RNA designed to direct site-specific base-pairing and cleavage at three GUC trinucleotide targets in the chloramphenicol acetyltransferase (CAT) mRNA. Other trinucleotides receptive to cleavage include GUA, GUU, UUC, CUC, AUC and AUU, conforming to the general rule NUX, where: N is any nucleotide; and X is A, C or U (Koizumi et al. 1989; Ruffner et al. 1989, 1990; Sheldon and Symons 1989; Perriman et al. 1992; Xing and Whitton 1992). Thus, by modifying the sequences flanking the catalytic domain, hammerhead ribozymes can be directed through base-pairing to receptive trinucleotide targets in any RNA species, cleave the RNA substrate and subsequently dissociate to catalyse further cleavage events.

Hammerhead ribozymes may prove to be powerful tools as regulators of gene expression and as therapeutic antiviral agents (Cameron and Jennings 1989; Cotten 1990; Rossi and Sarver 1990; Rossi et al. 1991). Ribozymes have recently been shown to restrict gene expression and replication of human immunodeficiency virus type 1 (HIV-1) in tissue culture (Dropulic et al. 1992; Sarver et al. 1990). However, effective strategies for the exogenous delivery of ribozymes into virus infected cells, either in the form of pre-formed synthetic RNA or

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amplifiable RNA expressed from eukaryotic RNA promoters, are required for their effective application as therapeutic antiviral agents. To address these problems we have constructed several ribozymes to three mRNAs of bovine ephemeral fever (BEF) virus. BEF virus is an insect-transmitted rhabdovirus that causes an acute debilitating disease of cattle and water buffalo in tropical and subtropical regions of Australasia, China and Africa. The genome of BEF virus comprises a 14.8 kb single strand of negative-sense RNA resembling that of other rhabdoviruses, with the exception that it possesses two rather than one glycoprotein gene as well as two or three small genes of unknown function (Walker et al. 1992).

Sequence analysis of cDNA clones covering most of the BEF virus genome has allowed the identification of potential ribozyme target sites. Ribozymes were designed to cleave at several positions in three selected mRNAs. Five ribozymes have been targeted at two large fragments of the low abundance L-gene mRNA encoding the viral RNA polymerase/replicase, two at the high abundance N-gene mRNA encoding the structural nucleocapsid protein and one at a putative non-virion (NV3)-gene mRNA of unknown function. Factors that affect the efficiency of *in vitro* cleavage of synthetic substrates, including temperature, substrate RNA secondary structures and reaction conditions were investigated together with ribozyme cleavage of native BEF virus mRNA *in vitro*. In addition, preliminary data will be reported on the effects of pre-formed synthetic ribozymes delivered into BEF virus-infected cells by transfection with cationic liposomes.

Materials and Methods

Plasmids, ribozymes and substrates

Ribozymes were cloned as synthetic single-stranded oligodeoxyribonucleotides by ligation into pGEM-3Z and transformation into *E. coli* SURE™ (Stratagene) host cells (Sambrook et al. 1989). The oligonucleotides incorporated partial *Eco*R1 and *Pst*1 sites, the ribozyme catalytic domain of the satellite RNA of tobacco ringspot virus (sTobRV) (Haseloff and Gerlach 1988) and 5′ and 3′ antisense BEF virus hybridisation arms of 8 to 11 nucleotides. The sequences of 8 BEF virus-specific ribozymes transcribed with T7 RNA polymerase from *Hind*111 linearised plasmid DNA are shown in Figure 1.

BEF virus cDNA was prepared from genomic and mRNA and cloned into pUC18 as described previously (Walker et al. 1992). Four cDNA inserts, m89 (3′ L-gene mRNA), m85 (5′ L-gene mRNA), m88 (N-gene mRNA) and g238 (NV3-gene mRNA)

```
6   5'-GGGCGAAUUCCUUU      ACCAAAUUCUGCAGGCAUGCA-3'  GUC
7   5'-GGGCGAAUUCCUCUAU    ACUUCAUCUGCAGGCAUGCA--3'  GUC
8   5'-GGGCGAAUUCCUUGUCUUU ACUGAAAACUGCAGGCAUGCA-3'  GUC
9   5'-GGGCGAAUUCUCU       ACUGUUGGCUGCAGGCAUGCA-3'  GUC
10  5'-GGGCGAAUUCUGCAGAGC  ACCUAAUCUGCAGGCAUGCA--3'  GUC
11  5'-GGGCGAAUUCGAUUGUU   ACUUCUAACUGCAGGCAUGCA-3'  GUC
12  5'-GGGCGAAUUCUAUAUGAU  AGAAUCUUCUGCAGGCAUGCA-3'  CUC
13  5'-GGGCGAAUUCAGGCCAUCU AACUUGAGCUGCAGGCAUGCA-3'  UUC
                      C    A
                   U        A
                  G          G
                 A  AG-G
                 UG U-A
                   C-G
                   C-G
                  G     A
                   U G
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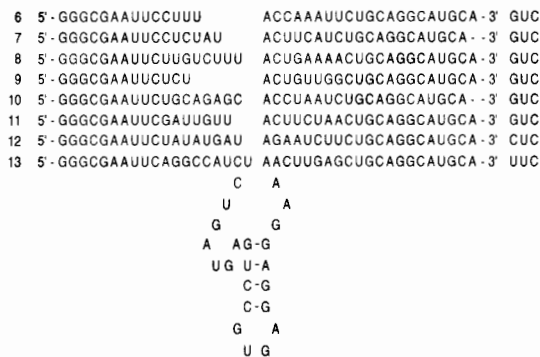
Fig. 1. Sequence of ribozyme RNAs transcribed with T7 RNA polymerase from pGEM-3Z linearised with *Hind*111. Nucleotides involved in base pairing with BEF virus mRNA sequences are underlined and the ribozyme catalytic domain common to all ribozymes is illustrated with ribozyme 13. The substrate trinucleotide sequences to which ribozyme cleavage is directed are indicated to the right.

were subcloned into either pGEM-4Z or pBluescript KS + in orientations to allow RNA transcription with T7 RNA polymerase. Several subclones of g238 were generated by digestion with *Eco*R1 and partial digestion with *Xho*11 or digestion with *Bam*H1 and partial digestion with *Bgl*11 and inserted into pGEM-4Z.

Computer analyses of RNA secondary structures

Secondary structure analyses of RNA transcripts were performed using the computer algorithm FOLDRNA and plotted using the SQUIGGLES program available on the Australian National Genomic Information Service (ANGIS) computing facility, University of Sydney.

PolyA + RNA isolation and purification from BEF virus-infected cells

The BSR line of BHK21 cells was infected with defective-interfering particle-depleted preparations of purified BEF virus at a multiplicity of infection (moi) of 5 plaque forming units (pfu)/cell and incubated at 37 °C until approx 1 + cytopathic effect (cpe) (16–20 hours). Total RNA was prepared from harvested cells and poly A + RNA purified on oligo-(dT) cellulose columns as described by Sambrook et al. (1989).

Ribozyme cleavage of synthetic and native BEF virus mRNA

Plasmid DNA was linearised with suitable restriction endonucleases and radiolabelled synthetic RNA transcripts prepared using T7, SP6 or T3 RNA polymerases (Promega) and [35S]-UTP according to

the manufacturer's instructions. Radiolabel incorporation efficiencies were determined by trichloracetic acid (TCA) precipitation to quantitate (pmoles) RNA synthesised, DNA was removed with RQ1 DNase (Promega) and the RNA precipitated with 2.5 M ammonium acetate and 2.5 volumes of ethanol. Synthetic RNA substrates and ribozymes were mixed, in some cases heated at 85 °C for 5 minutes, 50 mM Tris-HCl pH 7.4, 10 mM magnesium chloride was added and the reactions incubated at either 37 °C or 50 °C for 1 hour. Reactions were stopped by the addition of 50 mM EDTA pH 8.0 and an equal volume of 100% deionised formamide was added. Native BEF virus mRNA was treated with synthetic ribozymes as described above using 1–2 μg poly A+ RNA and 4 pmoles of ribozyme in a 5 μl reaction volume.

Electrophoresis and detection of ribozyme cleaved RNA

RNA was heated at 80–85 °C for 2–5 minutes and resolved in 0.4 mm thickness 5% polyacrylamide, 7 M urea, TBE (180 mM Tris-borate pH 8.3, 4 mM EDTA) gels. Radiolabelled RNA was detected by autoradiography and quantified using an AMBIS radioactivity imaging system. Poly A+ RNA was resolved in similar gels of 1.0 mm thickness and electroblotted under high field intensity (0.5 × TBE, 60 Volts, 1.1 Amps, 1 hour at 4 °C) onto positively charged nylon membranes. BEF virus mRNA-specific DNA probes were labelled with $[^{32}P]$-dCTP using the Multiprime DNA labelling kit (Amersham) according to the manufacturer's instructions. Membranes were hybridised with $2–4 × 10^5$ cpm/ml of $[^{32}P]$-labelled probe in QuikHyb™ solution (Stratagene) at 68 °C, 1 hour as described by the manufacturer and washed 2 × 15 minutes in 2 × SSC, 0.1% SDS at room temperature. Radiolabelled RNA was detected by autoradiography at −80 °C.

Liposome delivery of synthetic RNA ribozymes into BEF virus-infected cells

BHK21 cells were grown in 6-well plates (Costar; 9.6 cm²/well) to about 70% confluency and inoculated with approximately 2.5 pfu/cell of purified BEF virus. At 16–18 hours post-infection, the medium was removed and the cell monolayer washed with serum-free Basal Medium Eagles (BME) containing 14 mM HEPES pH 7.4, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. $[^{35}S]$-UTP-labelled ribozyme RNA (1.25 μg) was added to 10 μg Lipofectin™ Reagent (BRL) in 0.5 ml serum-free BME in a polystyrene tube (Corning), the mixture vortexed briefly and cells transfected for 5 hour

at 37 °C (Malone 1989). To label virus-induced proteins, the transfection medium was replaced with 0.5 ml/well RPMI 1640 without methionine and cystine (Flow Laboratories) supplemented with 2 mM L-glutamine and 50 μCi $[^{35}S]$-TRANS Label (ICN Biochemicals) and the cells incubated 37 °C for 30 minutes. Cells were washed with ice-cold phosphate buffered saline (PBS), solubilised in 150 μl electrophoresis sample buffer, the proteins resolved by SDS-polyacrylamide gel electrophoreses (Laemmli 1970) and detected by fluorography using Amplify™ reagent (Amersham).

Results and Discussion

In vitro ribozyme cleavage of synthetic BEF virus RNA

Synthetic BEF virus RNA substrates and ribozymes were transcribed with the appropriate RNA polymerases and pmoles of RNA produced determined from the relative incorporation of $[^{35}S]$-UTP. Substrate : ribozyme mixtures (ratio 0.5 : 3.0 pmoles) were prepared with or without pre-treatment at 85 °C, 5 minutes, 10 mM magnesium chloride was added and the reactions incubated at 37 °C or 50 °C for 1 hour. Electrophoretic analyses of cleavage of RNA transcripts from m85 with ribozymes 11 and 12, m88 with ribozymes 9 and 10 and m89 with ribozymes 6, 7 and 8 are shown in Figure 2. Cleavage products of the predicted sizes were obtained with all seven ribozymes. Quantification of cleavage efficiency using an AMBIS radioactivity imaging system indicated substrate catalysis levels of between 80% and 98% with no pre-heat step and incubation at 37 °C for 1 hour (data not shown). Preheating at 85 °C, 5 minutes to relax intermolecular ribozyme and substrate RNA secondary structures afforded little or no significant increase in cleavage efficiencies. Incubation at 50 °C resulted in visual decreases in uncleaved substrates suggesting greater ribozyme activity and/or turnover.

Effects of substrate RNA secondary structure on ribozyme cleavage efficiency

The significance of a substrate RNA secondary structure on ribozyme cleavage efficiency *in vitro* was investigated with ribozyme 13 targeted at the 5′ terminal region of the putative NV3-gene mRNA of BEF virus. Treatment of RNA transcribed from a cDNA clone (g238) of the NV3-gene with this ribozyme resulted in little cleavage (<5% at 37 °C and 50 °C) (Figure 3). After pre-heating 85 °C, 5 minutes and incubation at 50 °C for 60 minutes cleavage increased to 42%. A computer prediction
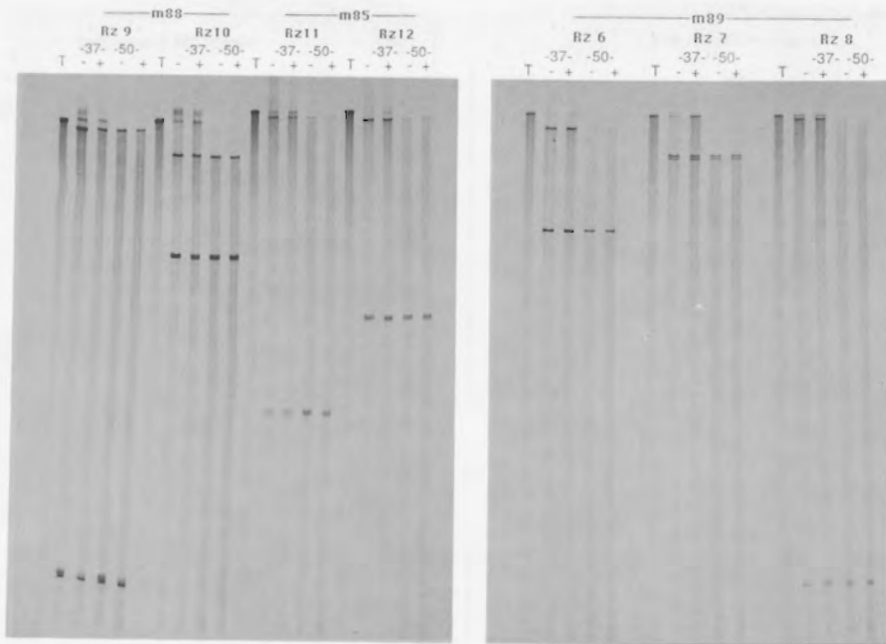
Fig. 2. Autoradiography demonstrating cleavage of synthetic RNA substrates of m89 with ribozymes 6, 7 and 8, m88 with ribozymes 9 and 10 and m85 with ribozymes 11 and 12. Reactions were incubated at 37 °C or 50 °C either with (+) or without (−) pre-heating at 85 °C, 5 minutes. RNA transcripts (T) and ribozyme-cleaved RNA were resolved on a 5% polyacrylamide, 7 M urea gel.

of the secondary structure of the RNA transcript revealed that the UUC target site resided within a 19 nucleotide stem loop structure with 13 base-paired nucleotides and a minimum free energy of − 13.2 kcal/mol (Figure 4). Therefore, several subclones were generated using *Bgl*11 and *Xho*11 restriction endonuclease sites that either removed or retained the stem loop in their predicted secondary structures (Figure 4). Significant ribozyme cleavage occurred (44% at 37 °C, 50% at 50 °C and 96% at 50 °C with pre-heating at 85 °C, 5 minutes) only with the RNA transcript g238 (X-E) (Figure 3) in which the stem loop was not predicted. Computer analysis of the putative native NV3-gene mRNA predicted a secondary structure with an identical stem loop to that of the g238 RNA transcript (data not shown). Additional experiments are required to determine whether ribozyme treatment of native mRNA *in vitro* will similarly result in minimal cleavage.

In vitro ribozyme cleavage of native BEF virus mRNA

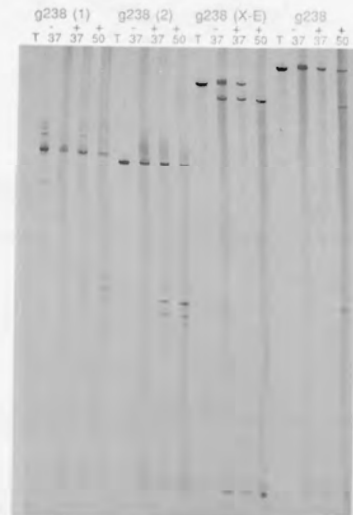PolyA + RNA from BEF virus-infected cells was treated with ribozymes and mRNA fragments

Fig. 3. Autoradiograph demonstrating cleavage of synthetic RNA substrates of g238 and subclones g238 (X-E), g238 (1) and g238 (2) with ribozyme 13. Reactions were incubated at 37 °C or 50 °C either with (+) or without (−) pre-heating at 85 °C, 5 min. RNA transcripts (T) and ribozyme-cleaved RNA were resolved on a 5% polyacrylamide, 7 M urea gel.
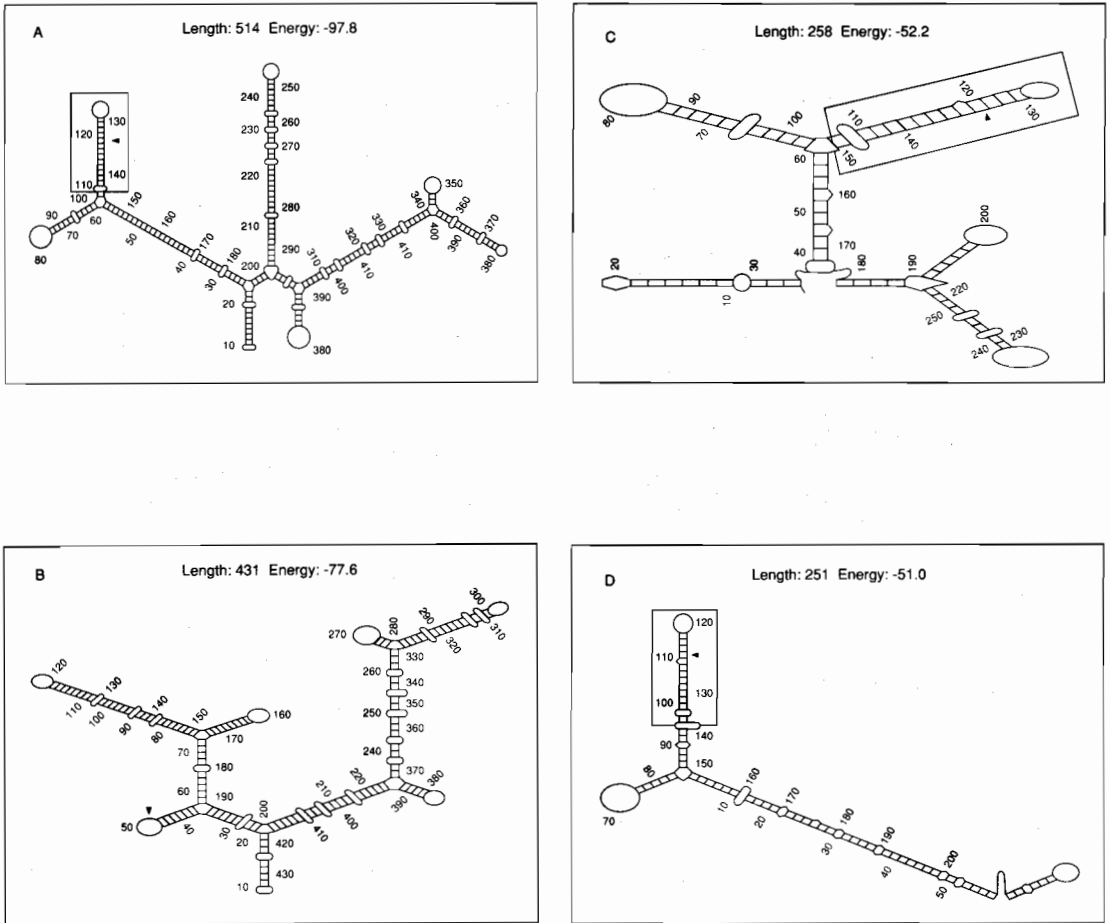
Fig. 4. Secondary structures of RNA transcripts of (a) g238 and subclones (b) g238 (X-E), (c) g238 (1) and (d) g238 (2) predicted by the computer algorithm FOLDRNA. Where present in a secondary structure, the stable stem loop (minimum free energy of − 13.2 kcal) containing the UUC trinucleotide sequence (   ) of ribozyme 13 is boxed.

Liposome delivery of ribozyme RNA into BEF virus-infected cells

The effects of pre-formed ribozyme RNA lipofected into BEF virus-infected BHK21 cells have been assessed initially under synchronous infection conditions (2.5–5.0 pfu/cell inoculum) at times post infection (16–18 hours) where host cell protein detected by hybridisation after electroblotting from polyacrylamide-urea gels. Cleavage of BEF virus N-gene mRNA with ribozymes 9 and 10 is shown in Figure 5. Reaction at 37 °C for 1 hour resulted in

significant cleavage with both ribozymes to generate fragments of the expected sizes. The larger 3′-terminal fragments were diffuse, suggesting heterogeneity in the polyA-tail lengths or greater susceptibility to degradation. Preheating at 85 °C for 5 minutes and incubation at 50 °C resulted in greater mRNA cleavage, suggesting that these conditions enhance ribozyme accessibility and/or turnover. Experiments to demonstrate ribozyme cleavage of native L- and NV3-gene mRNAs are currently in progress.
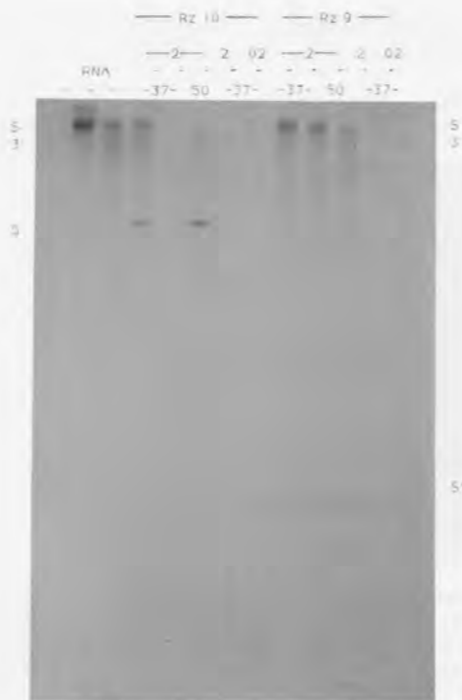
Fig. 5. Cleavage of BEF virus N-gene mRNA with ribozymes 9 and 10. Total polyA+ RNA (0.02, 0.2 and 2.0 μg) was mixed with 4 pmoles of ribozyme RNA and incubated in the presence of 10 mM MgCl₂ at 37 °C or 50 °C with (+) or without (−) pre-heating at 85 °C, 5 minutes. RNA was resolved on a 6% polyacrylamide/7M urea gel, electroblotted onto nylon membranes and detected by hybridisation with [^{32}P]-labelled m88 DNA and autoradiography.

synthesis was inhibited by virus-induced protein synthesis. The levels of [^{35}S]-methionine/cystine-labelled virus proteins synthesised with or without ribozyme treatment was determined by SDS-PAGE and fluorography (Figure 6). No discernible effects on virus protein synthesis occurred using this experimental approach with L-gene mRNA ribozymes 6 and 12 and N-gene mRNA ribozyme 9. However, [^{35}S]-labelled bands with estimated mol. wts. 19-23 kDa were observed that corresponded to the lipofected ribozymes. Although the data suggests that ribozymes transfected with cationic liposomes remain relatively stable under the experimental conditions employed in these analyses, it appears that transfection earlier in the infection cycle when virus-induced mRNAs are in considerably lower abundance will be advantageous in generating specific ribozyme inhibition of virus replication.

Potential application of ribozymes as therapeutic antiviral agents

The demonstration that hammerhead ribozymes specific to the L- and N-gene mRNAs of BEF virus cleave both synthetic and native mRNA targets *in vitro* affords some confidence in their potential to inhibit virus replication in an intracellular environment. The availability of several ribozymes directed at unique trinucleotide targets in different viral mRNAs may prove advantageous, as their simultaneous application should maximise inhibition of gene expression and circumvent potential problems associated with ribozyme accessibility at some substrate target sites. However, assessment of their antiviral activity will require effective strategies for the exogenous delivery of pre-formed ribozyme RNA into target cells. Preliminary data on the exogenous delivery of ribozymes with commercially available cationic liposomes has highlighted several important considerations. Firstly, that uncapped ribozyme RNA complexed with liposomes appears to be relatively stable, secondly, that lipofection efficiencies calculated from levels of cellular association of ribozymes suggest that > 100 000 molecules/cell are possible, and thirdly, that ribozyme treatment may not be advantageous during the exponential stage of virus replication when viral mRNAs are transcribed in abundance. Future experiments will focus on optimising lipofection efficiencies by quantifying ribozymes released into the cellular cytoplasm, identifying specific mRNA cleavage and determining effects on protein expression and virus yield in cells lipofected with ribozymes prior to infection with BEF virus.

Ultimately, the therapeutic application of ribozymes as antiviral agents will require stable, highly catalytic ribozymes and more sophisticated delivery approaches. With respect to exogenous delivery, the use of nuclease-resistant oligonucleotide analogs that retain ribozyme catalytic activity has attracted considerable interest (Cotten 1990; Rossi et al. 1991), as has the use of smaller RNA-DNA chimeric ribozymes or minizymes. Such pre-formed synthetic ribozymes might be targeted to specific cellular receptors via several approaches including antibody-coated liposomes (Leonetti 1991), proteoliposomes comprising virus fusion proteins (Gould-Forgerite et al. 1989) or conjugation to the 3′ or 5′ termini of lipophilic molecules such as cholesterol (Rossi et al. 1990).

The endogenous delivery of ribozyme RNA from DNA templates offers an alternative approach. Such DNA-based approaches may circumvent many of the difficulties associated with exogenous delivery of pre-formed ribozyme RNA. Several reports have demonstrated ribozyme-mediated gene regulation
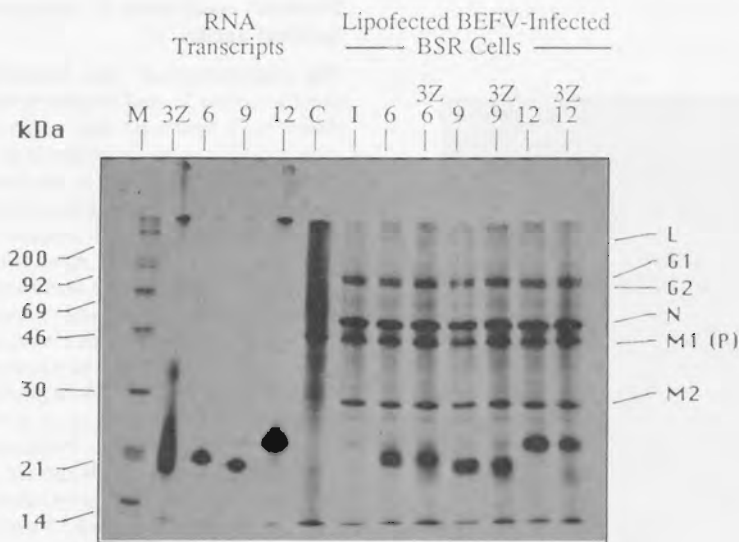
RNA Transcripts — Lipofected BEFV-Infected BSR Cells —

kDa M 3Z 6 9 12 C I 6 3Z/6 9 3Z/9 12 3Z/12

200 —
92 —
69 —
46 —
30 —
21 —
14 —

L
G1
G2
N
M1 (P)
M2

Fig. 6. Fluorograph demonstrating incorporation of [^{35}S]-methionoine/cystine into BEF virus-induced proteins in BHK21 cells lipofected with [^{35}S]-labelled RNA to ribozymes 6 and 12 directed to L-gene mRNA and ribozyme 9 directed to N-gene mRNA. RNA transcribed from pGEM-3Z linearised with *Hind*111 was employed as a carrier in separate transfections. [^{35}S]-labelled ribozyme and pGEM-3Z RNA transcripts were resolved to demonstrate the origin of 19-23 kDa bands in the BEF virus-infected BHK21 cells lipofected with ribozymes. M, [^{14}C]-labelled markers; C, uninfected BHK21 cells; I, BEF virus-infected BHK21 cell control.

and virus inhibition in cell culture either employing ribozyme RNA either transiently expressed from exogenously-delivered eukaryotic expression plasmids (Cameron and Jennings 1989) or synthesised in stably-transformed cell lines (Cotten 1990; Rossi et al. 1991).

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A Strategy for Purification and Peptide Sequence Analysis of Bovine Ephemeral Fever Virus Structural Proteins

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Abstract

A rapid and efficient procedure was devised for purification from virions of the bovine ephemeral fever virus (BEFV) G, N and M2 proteins. Purified virions were treated with 0.5% Triton X-100 to obtain a detergent-soluble fraction from which the BEFV G protein was purified by wheat germ lectin-affinity chromatography and size-exclusion high performance liquid chromatography (SE-HPLC). The Triton X-100 insoluble fraction was treated with 0.1% SDS and 0.05% RNase A and the BEFV N and M2 proteins were purified from the soluble fraction by SE-HPLC. BEFV proteins obtained by this procedure were suitable for endoproteinase digestion to generate peptides for amino acid sequence analysis. Peptides purified by 2 cycles of reverse-phase HPLC produced high quality amino acid sequence which confirmed sequences previously deduced from nucleotide sequence analysis of the corresponding viral genes.

BOVINE ephemeral fever (BEF) virus is an insect-borne rhabdovirus which has a structure similar to that of vesicular stomatitis virus (VSV) and rabies virus. Seen by electron microscopy, the virion is cone or bullet shaped, containing a helical nucleocapsid (Murphy et al. 1972). At the molecular level, the virus contains a 42S negative sense RNA genome and five structural proteins which, by analogy to rabies virus, have been named L (180 kDa), G (81 kDa), N (52 kDa), M1 (43 kDa) and M2 (29 kDa) (Della-Porta and Brown 1979, Walker et al. 1991). The L, N, M1 and M2 proteins are associated with a detergent-resistant nucleocapsid. The G protein is a detergent-soluble transmembrane glycoprotein which penetrates the lipid envelope to form spikes on the viral surface (Walker et al. 1991, 1992).

Various procedures have been described for the purification of rhabdovirus structural proteins for structure and function analysis, peptide mapping and amino acid sequencing. These include preparative isoelectric focusing (Dietzschold et al. 1978), immunoaffinity chromatography (Macfarlan et al. 1984), preparative SDS-polyacrylamide gel electrophoresis (Lafay and Benejean 1981) and ion-exchange chromatography (Bishnu and Banerjee 1984). High performance liquid chromatography (HPLC) has also been used in size-exclusion (SE) and ion-exchange chromatography to purify the G protein (Grassi et al. 1989) and N protein (Prehaud et al. 1990) of rabies virus.

In this paper, we describe a rapid and efficient HPLC procedure for the simultaneous purification of the G, N and M2 proteins of BEF virus in useful quantities and at a high level of purity. The suitability of the purified products for proteolytic digestion and peptide purification for subsequent amino acid sequence analysis is illustrated for both G and M2 proteins.

Materials and Methods

Virus Growth and Purification

The Australian BB7721 strain of BEFV was used. The virus was grown in 850 cm² roller bottle cultures of BHK-21 cells and purified from infected cell culture fluid by equilibrium density gradient centrifugation essentially as described by Walker et al. (1991).

Detergent Disruption of BEFV

The detergent disruption procedure was a modification of that described by Walker et al. (1991). Gradient purified virus was suspended in 0.5% Triton X-100, 10 mM Tris-HCl pH 7.4, rotated (end

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for end) at 6 rpm for 4 h at 25 °C and then centri-
fuged at 265 000 g for 15 min at 4 °C. The super-
natant fraction (SN1) was aspirated and retained.
The pellet was suspended in 0.1% SDS, 0.05%
RNase A, 100 mM Tris-HCl pH 7.4, rotated at 6 rpm
for 18 h at 25 °C and then centrifuged at 265 000 g
for 15 min at 4 °C. The supernatant fraction (SN2)
was aspirated and retained.

Lectin Affinity Chromatography

Lectin affinity chromatography was conducted using
a wheatgerm lectin-Sepharose column (38 mm ×
7 mm) as described by the manufacturer (Pharmacia)
pre-equilibrated with 50 mM Tris-HCl pH 7.4.
Bound fractions were eluted with 1 ml aliquots of
10% N-acetyl glucosamine (Sigma), 0.1% SDS,
50 mM Tris-HCl pH 7.4.

Size-Exclusion HPLC

Size-exclusion chromatography was conducted using
a Waters HPLC system consisting of a M510 pump,
a U6K injector and a M481 variable wavelength
detector coupled to a Shimadzu C-R3A Chroma-
topac integrator. Three chromatographic columns
connected in series included a Si200 Polyol (Serva
Chemicals) guard column (20 mm × 1.5 mm),
followed by 2 Protein Pak 300SW (Waters
Associates) 10 μm columns (300 mm × 7.8 mm).
Protein samples were concentrated to a total volume
of 250 μl using a Centricon 30 filter (Amicon) prior
to chromatography in 100 mM sodium thiocyanate,
0.1% SDS, 50 mM Tris-HCl pH 7.4 at 0.5 ml/minute
at a constant temperature of 30 °C.

Protease digestion, peptide purification and sequencing

To obtain peptides suitable for amino acid sequence
analysis, approximately 55 μg of the purified
glycoprotein G and 120 μg of M2 were reduced and
alkylated as described by Stone et al. (1989) followed
by digestion at 24 °C for 24 hours in 2 M urea,
100 mM ammonium bicarbonate pH 8.3. Digestion
was initiated by the addition of either endoproteinase
Glu-C for G protein or endoproteinase Lys-C for
M2, at a ratio of endoproteinase to protein of 1:40
(w/w). Peptides were purified by two cycles of
reverse phase HPLC on a Brownlee Aquapore
RP-300 C-8 column (Applied Biosystems Inc.).
Initial separation was conducted using a linear
gradient of 0–75% acetonitrile in 0.1% aqueous
heptafluorobutyric acid (HFBA). Selected peptides
were then applied to the same column and eluted in
a linear acetonitrile gradient in 0.1% aqueous
trifluoroacetic acid (TFA). The purified peptides
were sequenced using an Applied Biosystems pro-
tein sequencer.

SDS-polyacrylamide gel electrophoresis

Analytical gel electrophoresis of chromatographic
fractions was conducted in 6–18% gradient SDS-
polyacrylamide gels using the discontinuous buffer
system described by Laemmli (1970). Purified protein
fractions were analysed in 10% SDS-polyacrylamide
gels using the same buffer system. Staining of gels
was conducted by the modified silver staining method
of Rabilloud et al. (1988).

Protein estimations

Protein concentrations were estimated using: (i) BCA
protein assay reagent (Pierce); (ii) measurement of
absorbance at 280 nm compared to known amounts
of protein standard and assuming a specific absorp-
tion of 1 (g/l)-1 cm-1; and (iii) measurement of
fluorescence after reaction with o-phthaldehyde
(Viets et al. 1978; Goodno et al. 1981).

Results

Purification of the BEFV G, N and M2 Proteins

The strategy adopted for purification of the G, N
and M2 proteins is illustrated in Figure 1. Gradient
purified virus was prepared and treated with 0.5%
Triton X-100 to disrupt the lipid envelope. The
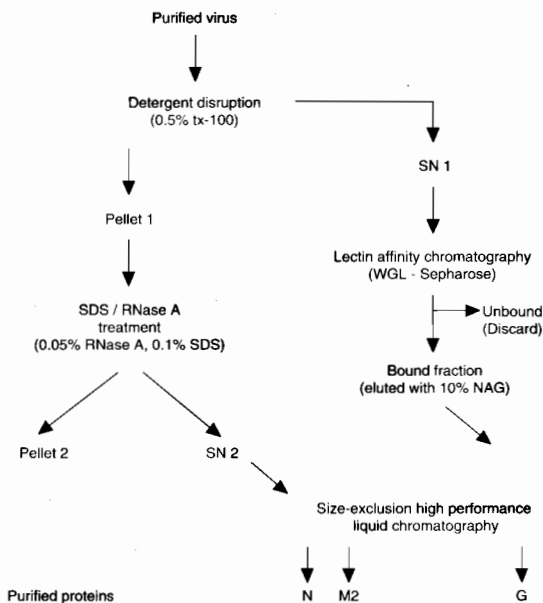detergent-soluble fraction (SN1) contained the BEF

Fig. 1. Procedure for purification of N, M2 and G pro-
teins of BEF virus.

virus G protein, a residue of bovine serum albumin (BSA) carrier protein and contaminating cellular proteins associated with the purified virus. The G protein was purified from the SN1 fraction by lectin-affinity chromatography and size-exclusion HPLC. After lectin-affinity chromatography, the bound fraction contained primarily the BEF virus G protein and several contaminating cellular glycoproteins in the size range 100 kDa–150 kDa. These could be resolved by size-exclusion HPLC in which the G protein had a distinctive retention time of 22.65 minutes (Figure 2A).

After treatment of the purified virus with Triton X-100, the detergent-insoluble fraction (PELLET 1) contained the L, N, M1 and M2 proteins. These were solubilised by treatment with SDS and RNase A to generate a fraction (SN2) from which all were purified by SE-HPLC. The L and M1 proteins were relatively minor components which were clearly resolved but were difficult to obtain in quantities

Fig. 2. Size exclusion HPLC chromatographic profiles of BEFV G, N and M2 proteins. (A) Chromatography of the WGL-Sepharose bound fraction from which the G protein peak eluted at 22.65 minutes. (B) Chromatography of the soluble fraction (SN2) after treatment of virions with Triton X-100, SDS and RNase A. Elution times for N, M2 and RNase A peaks were 23.06, 26.70 and 31.31 minutes respectively. Small amounts of the BEF virus L protein were also detected in the peak which eluted at 19.53 minutes. The BEF virus M1 protein eluted in the area between N and M2.

suitable for amino acid sequence analysis. The N and M2 proteins were major components which were obtained in quantity from fractions with distinctive retention times of 23.06 and 26.70 minutes respectively (Figure 2B).

The purified protein fractions were compared with the gradient-purified virus preparation by polyacrylamide gel electrophoresis under reducing conditions, followed by silver staining. As shown in Figure 3, the G and M2 proteins resolved as single bands and contained no detectable contaminating proteins. The identity of each of these proteins was confirmed by immunoblotting using G protein-specific and M2 protein-specific monoclonal antibodies (not shown). The N protein resolved as several associated bands under non-reducing conditions as shown in Figure 3. These appeared to be conformational isomers of the N protein as they resolved as a single band under reducing conditions and each reacted in immunoblots with N protein-specific monoclonal antibody (not shown).
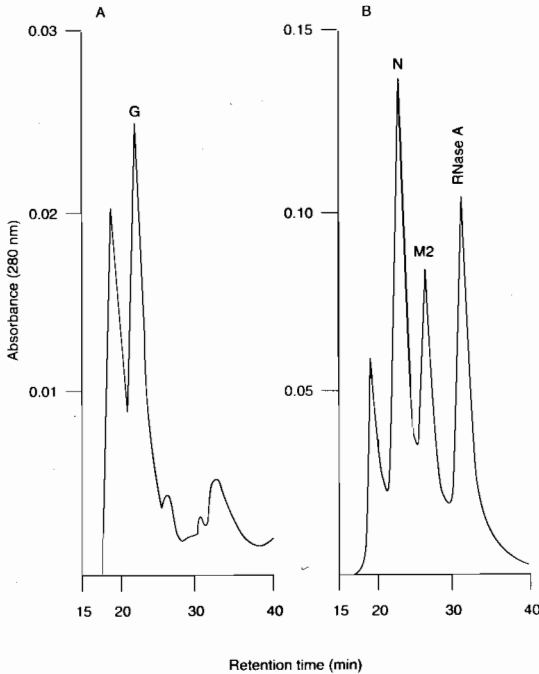
Protease digestion and amino acid sequencing of the G and M2 proteins

The purified preparations of the G and M2 proteins were digested with endoproteinase Glu-C and endoproteinase Lys-C respectively, and the resulting peptides were separated by 2 cycles of reverse phase HPLC. Each protein generated several unique peptides in sufficient quantity for amino acid sequence analysis. The sequences of a selected set of peptides derived from each protein are shown in Figure 4. The identity of the peptides was confirmed by comparison with amino acid sequences deduced from the known nucleotide sequences of the G and M2 genes. N-terminal sequence analysis of the N protein preparation indicated that, like the N proteins of rabies virus and VSV, the BEF viral N protein has a blocked N terminus. Conditions for endoproteinase digestion of the N protein are presently being optimised, so internal amino acid sequence can be obtained.

Discussion

The procedure presented in this paper allows the successful purification of the major structural proteins of BEF virus G, N and M2, in sufficient quantities and purity to facilitate the production of useful internal amino acid sequence. In a typical preparation, 2 mg of purified virus, on average yielded 69 μg G protein, 192 μg N protein and 120 g M2 protein. Small amounts of L and M1 proteins, which are minor components of the virion, are also purified during this procedure. However, additional preparations will be required to obtain sufficient material
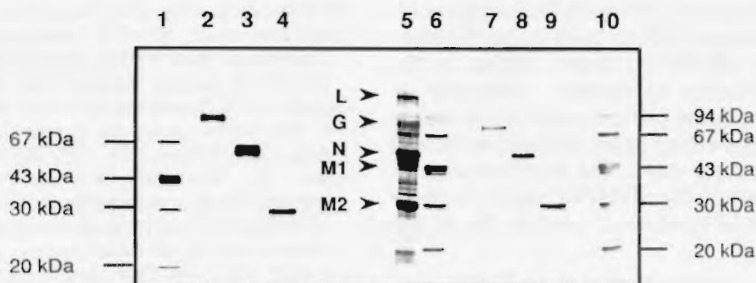
Fig. 3. SDS-PAGE of purified G, N and M2 proteins. The gel on the left illustrates electrophoresis under non-denaturing conditions of purified G (lane 2), N (lane 3) and M2 (lane 4) proteins. The gel on the right illustrates the electrophoresis of purified BEF virus (lane 5), and purified G (lane 6), N (lane 7) and M2 (lane 8) proteins, under denaturing conditions in the presence of 10 mM dithiotreitol. Molecular weight marker proteins were run in lanes 1, 6 and 10.

Peptide	Amino acid sequence
G141a	(E) K I Y N V P V N C G E
G141b	(E) C I T V K S F R S E
G163	(E) K L C L S L P D S X R V X X D C N I
G164	(E) X W Y F X T C I E
M1109	(K) V L D V M V D E Y D G S Y L S
M1303	(K) N I L D A Y N L E L G N G S K
M1702	(K) Y N N G F T E V I E F T G T A E I H P R D Q E
M1903	(K) A L I I T S Y L T I G T H L R R M M S S

Fig. 4. Amino acid sequences of G and M2 peptides. Purified G and M2 proteins were digested by endoproteinase Glu-C and endoproteinase Lys-C respectively. The one letter code for amino acids has been used. Residues which could not be assigned with confidence are represented by an X. The initial (E) or (K) are assumed to be present because of the specificity of the respective endoproteinases.

for further analysis. SE-HPLC purified G and N proteins have been used successfully for vaccination trials in cattle, (Uren et al. these proceedings).

High performance liquid chromatography is not new in the field of virus structural protein purification, but this technology has not been employed widely in rhabdovirus protein purification protocols. By using the powerful resolving power of SE-HPLC we have achieved the purification of all 5 BEF virus structural proteins from one preparation. Our initial attempts at simultaneous purification of all virion proteins by SE-HPLC were hindered by a number of complicating factors. Firstly, a residue of growth medium BSA carrier protein and contaminating cellular proteins are consistently present in the solubilised virus fraction. The complexity of this fraction is illustrated in Figure 3, lane 5. In SE-HPLC, BSA coelutes and contaminates both G and N proteins and is difficult to remove completely. Secondly, resolution of proteins on SE-HPLC depends primarily on molecular weight and shape of the molecule. The glycoprotein G, behaves irregularly under SE-HPLC conditions in 0.1% SDS and has a high retention time than would be predicted from the molecular weight. However, N protein elutes where expected, resulting in poor resolution between the N and G proteins. This is illustrated in Figure 2 where G and N proteins are chromatographed under identical conditions and the elution times are closer than expected at 22.65 and 23.06 minutes respectively. To overcome this problem, the G protein was initially solubilised by mild disruption of the virus lipid envelope with 0.5% Triton X-100. In addition to the G protein, the soluble fraction contained contaminating non-viral components originating from culture cells and growth media. Wheat germ lectin-affinity chromatography provided a simple and efficient method for semi-purification of the G protein from the majority of contaminating substances. Subsequent resolution of the G protein from the other glycoproteins was achieved by SE-HPLC. Disruption of the detergent-insoluble fraction resulted in good resolution of N, M2 and contaminating RNase A by SE-HPLC. A second cycle of SE-HPLC was sometimes necessary to achieve the required level of purity.

The SE-HPLC system employed in this paper has been used previously at this laboratory during the isolation and purification of membrane proteins of

the cattle tick *Boophilus microplus* (Willadsen et al. 1989). The inclusion of SDS in both the preliminary buffers and the SE-HPLC eluent, assists in the prevention of protein aggregation, especially of glycosylated membrane proteins, and in the reduction of protein loss during pellet resolublisation and sample concentration steps. The incorporation of sodium thiocyanate in the SE-HPLC eluent, appears to improve protein resolution, possibly by an ion pairing effect.

An important objective of our work has been nucleotide sequence analysis of the BEF virus genome. The assignment of gene sequences to particular structural proteins has been possible when there was highly significant amino acid sequence homology with proteins of related rhabdoviruses. For other proteins, amino acid sequence information obtained from peptides of HPLC purified proteins, has provided definitive identification. Sequence of peptides from both G and M2 have confirmed sequences previously deduced from sections of the corresponding gene. Sequence of peptides from virion G protein has allowed the differentiation of genes encoding the structural (G) and non-structural (GVS) glycoproteins of BEF virus (Walker et al. 1992).

Acknowledgments

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Expression in *Eschericia coli* of the Nucleoprotein (N) and Glycoproteins (G and GNS) of Bovine Ephemeral Fever Virus

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Abstract

Copies of genes encoding the bovine ephemeral fever virus glycoprotein (G), nonstructural glycoprotein (GNS) and nucleoprotein (N) were inserted into pGEX plasmid vectors. These plasmids direct the synthesis of foreign polypeptides as fusions with a 26 kD portion of glutathione S-transferase. The N protein was expressed in large quantities as a near full-length fusion protein and as amino and carboxy-terminal fragments. A group reactive N protein epitope was located in the carboxy domain. The carboxy-terminal fragment was partially soluble and could be purified by affinity chromatography. The full-length fragment was soluble in 6 M urea and could be purified by Fast Protein Liquid Chromatography. The G protein was expressed at low levels with or without the putative signal peptide sequence. The GNS protein was expressed at moderate levels and was insoluble. G protein monoclonal antibodies were used to identify two non-conformational, neutralising epitopes which remain active in the expressed G protein. Cross-reaction with these monoclonal antibodies was evident with the GNS protein, suggesting conservation of these epitopes between the glycoproteins.

THE bovine ephemeral fever (BEF) virus glyco-protein (G) is an 81 kD protein located on the virion surface (Della-Porta and Brown 1979; Walker et al. 1991). The G protein is the only virion protein found to induce neutralising antibodies (Cybinski et al. 1990) and has been shown to confer protection in cattle (our unpuplished data). Recently a gene encoding a non-structural glycoprotein has been reported (Walker et al. 1992) which codes for a 90 kD protein (GNS). The antigenicity of this protein is unknown, but like the G protein, it contains a trans-membrane domain and is likely to be abundantly expressed on the surface of BEF virus infected cells and may therefore be an important immunogen. The nucleoproteins (N) of related rabies virus and vesicular stomatitis virus are known to have an important role in cell mediated immunity (Fu et al. 1991; Van Bleek and Nathenson 1990) and it is possible that the BEF virus N protein will also aid in inducing protective immunity.

This paper describes the investigation of the pro-duction of recombinant BEF virus G, GNS, and N proteins in *E. coli*. Expression was effected by cloning of the corresponding genes into pGEX plasmid vectors which allow expression of foreign proteins linked with glutathione-S-transferase (Smith and Johnson 1988). We also report preliminary analysis of the antigenic structure of these recombi-nant proteins.

Materials and Methods

Cloning and selection of recombinants

Clones containing the complete coding regions of the G protein gene (G1.A6) and the GNS protein gene (G2.C1) were described by Walker et al. (1992). These clones were digested with *Bgl* II and *Bam* H1 respectively before subcloning the cDNA inserts into the *Bam* HI site of pGEX-2T. Clones of the G pro-tein gene from which the signal peptide coding region was deleted were obtained by polymerase chain reaction (PCR) amplification from G1.A6 by using the PCR primer pairs G1.1D(5'GGCCAGATCT AAGATTCATTTGGAGA-AAATTTAC3')/ G1.2B(5GGCCAGATCTTTAATGATCAAAGA ACCTATCATCACCAG 3') The PCR products were extracted once with phenol/chloroform and

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then digested with *Bgl* II before cloning into the *Bam* HI site of pGEX-2T. A near full-length clone of the N gene (pUCm88.3) was used for subcloning into pGEX. Partial digestion of pUCm88.3 with *Bam* HI and *Eco* RI generated a full-length (1334 base) insert and small (565 base) and large (769 base) fragments. The full-length insert and the small fragment were subcloned into the *Bam* HI/*Eco* RI site of pGEX-3X. The large N gene fragment was cloned into the *Eco* RI site of pGEX-1. Clones selected to contain inserts of the expected size were sequenced to confirm correct orientation using T7 DNA polymerase (Sequenase version 2.0, United States Biochemicals) according to the manufacturers modifications of the dideoxy-chain termination method of Sanger et al. (1977) and pGEX forward and reverse sequencing primers (CSIRO).

Expression of fusion proteins and isolation of inclusion bodies

The expression of fusion proteins was induced using IPTG essentially as described by Smith and Johnson (1988). One tenth volume of overnight culture was inoculated into an appropriate volume of TYM containing 50 μg/ml ampicillin. The culture was incubated vigorously at 37 °C until an A_{600} of 0.6 to 1.0 was reached. The culture was incubated for an additional 3–16 hours after addition of IPTG to 0.2 mM. Bacterial cultures were then harvested by centrifugation at 5000 g for 10 minutes. Cells were resuspended in lysis buffer (50 mM Tris HCl pH 8.0, 50 mM NaCl, 1 mM EDTA, 1 mM DTT and 1 mM PMSF) and disrupted either by sonication 2 × 10 minutes in water bath sonicator or by treatment with lysozyme/sodium deoxycholate (Sambrook et al. 1989). Inclusion bodies were prepared from the lysate as described by Marston et al. (1984). Both soluble and insoluble fractions were retained for later analysis.

Fusion protein purification

Inclusion bodies were solubilised in lysis buffer containing 8 M urea. Samples were purified by Fast Protein Liquid Chromatography (FPLC) (Waters 650E) through Sepharose 12A at 216 psi in 25 ml bed volume with a flow rate of 0.5 ml/minute. 500 μl fractions were collected, analysed by SDS-polyacrylamide gel electrophoresis (PAGE) and peak fractions pooled.

Affinity purification of the fusion proteins was conducted essentially as described by Smith and Johnson (1988). Supernatant from the *E. coli* lysate was prepared as described and mixed at room temperature for 20 minutes with approximately 1/10 volume of 50% glutathione agarose beads (Sigma).

Following adsorption, the beads were collected by brief centrifugation at 500 g and washed twice with 10 volumes of TBS (100 mM Tris-HCl pH 7.4, 50 mM NaCl), 0.1% Triton X-100, 0.1% Tween-20, 1 mM DTT and once with TBS. The fusion protein was eluted from the beads by washing 1 x 5 minutes with 2 bead volumes of 50 mM Tris HCl (pH 8.0) containing 5 mM reduced glutathione (Sigma).

SDS-polyacrylamide gel electrophoresis and immunoblotting

SDS-PAGE was conducted using the discontinuous buffer system of Laemmli (1970). Following electrophoresis, gels were stained with Coomassie brilliant blue (R-250) or transferred to nitrocellulose membranes (Amersham) by electroblotting (Towbin et al. 1979). Immunodetection was conducted using mouse monoclonal antibodies as described by Walker et al. (1991).

Results

Expression of the BEF virus G protein

A full-length DNA copy of the BEF virus G protein gene was cloned into the multiple cloning site of pGEX-2T. Five selected clones were confirmed to be in the correct orientation and reading frame by nucleotide sequence analysis. Expression of GST-fusion proteins was induced in each clone using IPTG and cell lysates were prepared and analysed by SDS-PAGE. As illustrated in Figure 1 for clone G1.24, induction resulted in expression of a 94 kD protein, corresponding in size to that expected for a full-length GST-fusion protein. The protein was expressed at low levels in each of the full-length clones. To improve the level of expression, the hydrophobic signal peptide domain was removed from the G protein gene and the truncated gene was subcloned into the multiple cloning site of pGEX-2T. Several clones confirmed to have inserts in the correct orientation and reading frame were tested for fusion protein expression. An induced fusion protein was detected with slightly lower molecular weight than the 94 kD fusion protein but no enhancement of the level of expression was apparent.

The antigenicity of the fusion products was analysed by immunoblotting with BEF virus G protein neutralising monoclonal antibodies. Monoclonal antibodies 17B1 and 5A5, directed at non-conformational antigenic sites G1 and G4 respectively, reacted specifically with the 94 kD protein and with several bands of lower molecular weight which appeared to represent fragments or incompletely transcribed fusion products (Figures 2 and 3). Removal of the signal peptide did not change the reactivity with the monoclonal antibodies.

G FUSION PROTEINS

G1.24 G1.6B G2.14
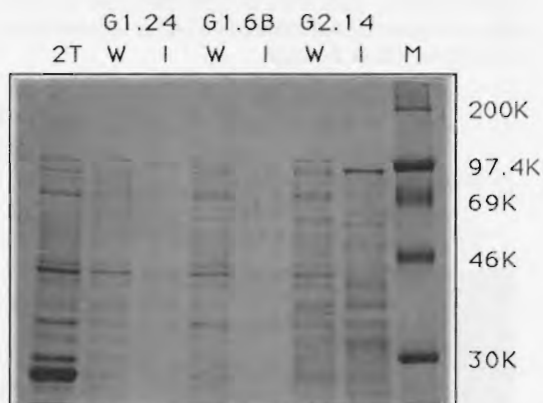2T W I W I W I M

200K
97.4K
69K
46K
30K

Fig. 1. Coomassie stained SDS-polyacrylamide gel of total *E. coli* proteins (W) and isolated inclusion bodies (I) from clones G1.24, G1.6B and G2.14; total cell proteins from *E. coli*/pGEX-2T (2T); protein standards (M). Molecular weights are indicated on the right.

IMMUNODETECTION 17B1
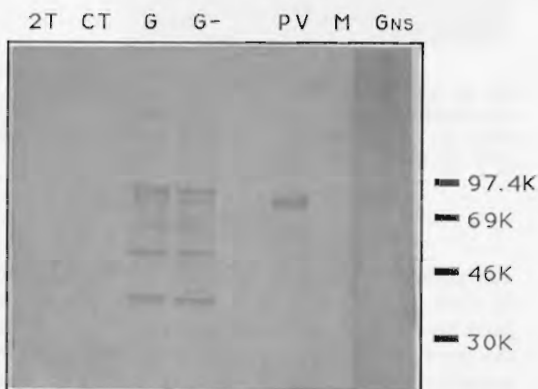
2T CT G G- PV M GNS

97.4K
69K
46K
30K

Fig. 2. Immunodetection with monoclonal antibody 17B1 of isolated inclusion bodies from *E. coli*/pGEX-2T; clones G1.18 (CT), G1.24 (G), G1.6B (G-), G2.14 (GNS); BEF purified virus (PV); protein standards (M). Molecular weights are indicated on the right.

Expression of BEF virus GNS protein

Four full-length clones of the BEF virus GNS protein gene in pGEX-2T were selected and confirmed in the correct orientation and reading frame. As illustrated for clone G2.14, IPTG induction resulted in moderate expression a 90 kD GST-fusion protein which corresponded in size to that expected for a full-length product (Figure 1). Immunoblotting of the induced proteins with BEF virus G protein monoclonal antibodies 17B1 and 5A5 indicated that the 90 kD fusion protein reacted weakly but specifically with each. No lower molecular weight fragments or abortive products were detected. The results indicated that the BEF virus G and GNS proteins share non-conformational epitopes which remain active in the expressed GST-fusion products.

Expression and purification of the BEF virus N protein

A near full-length copy of the BEF virus N protein gene and two fragments, derived by cleavage of the gene at a single internal *Eco* R1 restriction site, were cloned into the multiple cloning site of pGEX expression vectors. pGEX clone m88.3 contained the near full-length N protein gene. Clone m88.1 contained a 565 nucleotide N protein gene fragment encoding the NH_2-terminal N protein domain. Clone m88.15 contained a 769 nucleotide N protein gene fragment encoding the COOH-terminal N protein domain. As illustrated in Figure 4, all three clones expressed GST-fusion proteins in abundant quantities. The 46 kD fusion protein expressed by clone m88.15 was partially soluble in low salt buffers and could be purified by affinity chromatography using glutathione-agarose. The 60 kD and 30 kD fusion proteins expressed by clones m88.3 and m88.1 respectively were insoluble in low salt buffers but could be solubilised using 8 M urea and partially purified by FPLC (Figure 5).

IMMUNODETECTION: 5A5
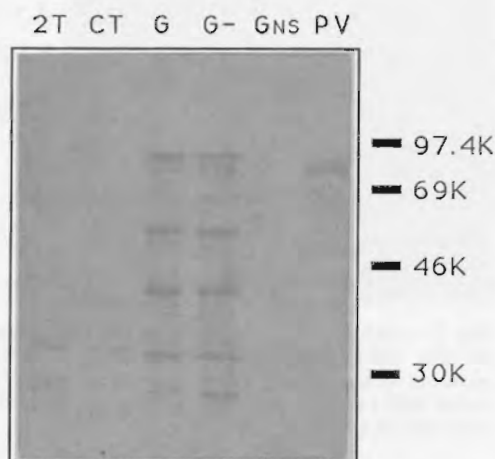
2T CT G G- GNS PV

97.4K
69K
46K
30K

Fig. 3. Immunodetection with monoclonal antibody 5A5 of isolated inclusion bodies from *E. coli*/pGEX-2T; clones G1.18 (CT), G1.24 (G), G1.6B (G-), G2.14 (GNS); BEF purified virus (PV); protein standards (M). Molecular weights are indicated on the right.
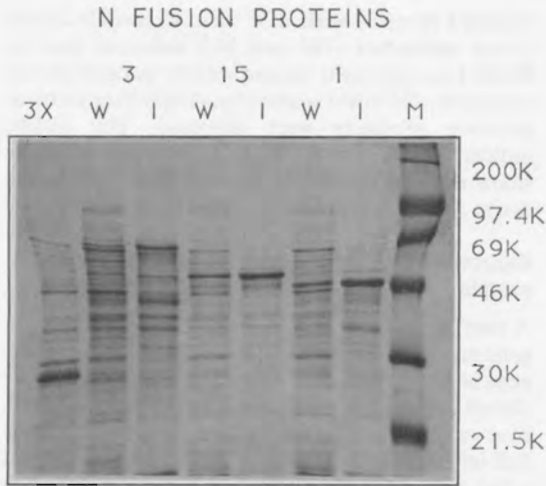
Fig. 4. Coomassie stained SDS-polyacrylamide gel profile of total cell proteins (W) and isolated inclusion bodies (I) from *E. coli*/pGEX-3x; clones m88.3 (3), m88.15 (15) and m88.1 (1). Protein standards (M). Molecular weights are shown on the right.
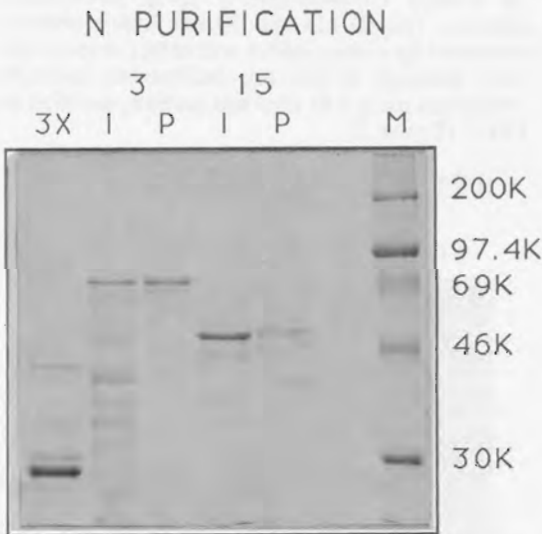

Fig. 5. Coomassie stained SDS-polyacrylamide gel profile of total cell proteins from *E. coli*/pGEX-3x; Isolated inclusion bodies (I) and FPLC purified proteins (P) of clones m88.3 (3) and m88.15 (15). Molecular weights are indicated on the right.

All three expressed N-fusion proteins were tested by protein immunoblotting for reaction with BEF virus N protein monoclonal antibody 11A3 (Figure 6). Strong reactions were detected with both the near full-length and COOH-terminal fusion proteins but no reaction was detected with the NH_2-terminal fusion protein, indicating that the antigenic site identified by monoclonal antibody 11A3 is located in the COOH-terminal domain.
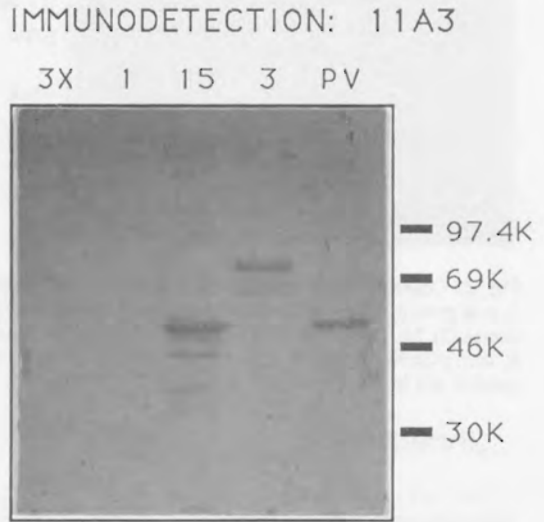
Fig. 6. Immunodetection with monoclonal antibody 11A3 of isolated inclusion bodies from; *E. coli*/pGEX-3x; clones m88.1 (1), m88.15 (15) and m88.3 (3); purified BEF virus (PV). Molecular weights are indicated on the right.

Discussion

This paper describes the expression of the BEF virus G, G_{NS} and N proteins in *E. coli* as GST-fusion proteins and examines aspects of the antigenicity of the expressed proteins. The level of expression of the proteins varied. The N protein and NH_2- and COOH-terminal fragments of the N protein were all expressed abundantly, the G_{NS} glycoprotein was expressed at a lower but useful level, and the G glycoprotein was poorly expressed. The reason for the lower levels of expression of the G and G_{NS} proteins is not clearly established but the presence of hydrophobic domains is known to reduce expression levels in *E. coli*, particularly of glycoproteins (Sisk et al. 1992). For the vesicular stomatitis virus (VSV) G protein it has been reported that the hydrophobic signal peptide domain is deleterious to expression in *E. coli* and efficient expression of the rabies virus G protein has been reported when the signal peptide is removed. However, removal of the signal peptide domain from the BEF virus G protein gene did not improve the poor level of expression. It is possible

that the strongly hydrophobic transmembrane domains of the BEF virus glycoproteins are influencing the expression levels.

One potential application of the *E. coli* expressed BEF viral proteins is in the development of a recombinant vaccine. The BEF virus G protein purified from virions has been shown to protect cattle against experimental BEF infection (our unpublished data) and it is possible that the GNS and N proteins may have a role in inducing protective immunity. As a transmembrane glycoprotein, the GNS protein may be located on the surface of BEF virus-infected cells during natural infections and provide a target for antibody-mediated cytolytic immune mechanisms (Walker et al. 1992). The BEF virus N protein, like those of VSV and rabies virus (Fu et al. 1991), may have a role in stimulating cell-mediated immunity by presentation of peptides on the cell surface in association with class 1 Major Histocompatibility Complex (MHC) molecules. In a preliminary analysis of the antigenic structure of the expressed proteins, we have tested the reactivity of a selected panel of BEF virus monoclonal antibodies directed at non-conformational antigenic sites. The N protein antigenic site defined by monoclonal antibody 11A3 remained intact in both the near full-length N-fusion protein and the COOH-terminal fragment, locating the site in the COOH-terminal domain. Two non-conformational antigenic sites (NI and NIII) have been located in the corresponding region of the rabies virus N protein but their role in protective immunity has not been defined. For VSV, the major N protein cytotoxic T cell epitope is located at amino acids 52–59 in the NH_2-terminal domain (Van Bleek and Nathenson 1990).

G protein neutralising antigenic sites G1 and G4 also appeared to remain intact in the *E. coli*-expressed fusion protein, suggesting that N-glycosylation of the G protein in eukaryote cells does not contribute to the essential structure of these sites and that the expressed G protein would induce neutralising antibodies. The G1 and G4 sites were also detected in the expressed GNS-fusion protein establishing the existence of a serological relationship between the two BEF virus glycoproteins. As far as we are aware, this represents the first evidence of a serological relationship between two distinct proteins encoded in the same virus and highlights the potential importance of the GNS protein in serological diagnosis and protective immunity.

We have described procedures for the purification of fusion proteins containing near full-length N protein and NH_2 and COOH-terminal N protein fragments are also described. Isolation of inclusion bodies was the most significant stage of the purification procedure, further purification by FPLC increased the purity only slightly. Purification by affinity chromatography gave a very pure product but at low yields. The production of this synthetic N protein will allow for further analysis of its antigenic structure and suitability as a candidate vaccine. Similarly further analysis of the antigenic structure of the G and GNS proteins will allow evaluation as potential for vaccine antigens.

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Walker, P.J., Byrne, K.A., Cybinski, D.H., Doolan, D.L. and Wang, Y. 1991. Proteins of bovine ephemeral fever virus. Journal of General Virology, 72, 67–74.

Vaccines and Antigenic Variation

Inactivated mouse brain and cell culture vaccines for ephemeral fever have been used in South Africa, Japan, Australia and China for some time. Although these vaccines provide a degree of protection it has been recognised that better vaccines are needed. Following the methodology used with other rhabdoviruses, such as vesicular stomatitis and rabies viruses, it has now been demonstrated that sub-unit vaccines, based on protective enveloped glyco-proteins, can protect cattle against ephemeral fever. The papers in this section will detail some of the research on sub-unit vaccines which has been carried out in China and Australia.

Until recently it was thought that there were no viruses which were closely related to BEF virus and that all isolates of BEF virus from Africa, Japan and Australia were identical. It is now known that there are a group of related rhabdoviruses of which BEF virus is a member. The existence of these viruses has implications for diagnosis and vaccination as is discussed in this section. In Australia, studies with monoclonal antibodies have also shown that varia-tion in BEF virus does occur over time and that not all isolates are identical. To what extent this is important for vaccine development is not yet clear and further studies need to be undertaken in other countries where BEF virus infection occurs.

The vaccine consisted of the following components: diluted V antigen; and Freund's complete adjuvant (FCA) or white oil adjuvant consisting of a mixture of white oil (light mineral oil, A grade), span 85 (sorbitan triolate) and Tween 85 (polyoxyethylene 20 sorbitan triolate) at a ratio of 90:7:3. Vaccine emulsions were prepared in an homogeniser by adding various diltions of V antigen to equal volumes of either FCA or white oil adjuvant. The emulsions were then stored at 4 °C.

Cattle used in the vaccination trial were injected subcutaneously on two occasions, three weeks apart. On days 22, 29, 44 and 80 following the second injection, animals were challenged by intravenous injection of 0.5 ml of blood containing virulent virus. Serum samples for serological testing were collected at immunisation and challenge. All control animals were immunised with the C antigen using the same protocol.

To determine the duration of immunity following vaccination, a group of cattle were vaccinated with 20-fold diluted vaccine (9015 and 9020) mixed with white oil adjuvant. Different animals were challenged at 1, 3, 6 and 9 months after vaccination. In another experiment, the effect of vaccine storage was evaluated by vaccinating different groups of cattle with vaccine which had been stored at 4–8 °C for 1, 2 or 4 months. The vaccine was also evaluated in a field trial using cattle on two farms in

Guangdong Province, and one farm in Shandong Province.

Results

The V antigen was inoculated onto confluent monolayers at BHK21 cells and observed for six days. There was no evidence of virus growth. Adult mice and guinea pigs inoculated subcutaneously and intraperitoneally with V antigen showed no adverse reactions. No virus could be recovered when V antigen was inoculated intracerebrally into suckling mice and given one or two blind passages.

Of 16 cattle vaccinated with FCA vaccines, five showed a transient rise in temperature above 40 °C following challenge with virulent virus. Local tissue reactions were found at the injection sites of 14 cattle. From two to seven months after injection, swelling was still present but had diminished. Of 14 cattle vaccinated with white oil adjuvant vaccines, four showed a transient rise in temperature and eight had local swelling similar to cattle receiving FCA vaccines. However, by 20 days after injection, the swelling had markedly decreased or disappeared. There was no tissue reaction in any cattle at two months after injection.

The results of the immunity tests are presented in Table 1. The challenge virus produced clinical BEF

Table 1. Results of vaccination of cattle with a BEF virus glycoprotein vaccine.

Experiment no.	Vaccine batch no.	Adjuvant	Vaccine volume (ml)	Vaccine dilution	Challenge day[1]	No. protected/ no. challenged
1	85	FCA	2	1/1	44	3/3
	85	FCA	2	1/20	44	3/3
	Control	—	—	—	—	0/3
2	86	FCA	1	1/1	80	2/2
	86	FCA	1	1/20	80	1/3
	Control	—	—	—	—	1/4
3[2]	86	FCA	2	1/20	29	2/2
	86	WOA	1	1/1	29	3/3
	86	WOA	2	1/20	29	3/3
	Control	—	—	—	—	1/3
4	87	WOA	1	1/1	22	2/2
	87	WOA	2	1/20	22	3/3
	87	WOA	2	1/40	22	3/3
	Control	—	—	—	—	0/3

[1] Days post-second vaccination
[2] Vaccine stored for 12 months before use
FCA = Freunds complete adjuvant
WOA = White oil adjuvant
— = not applicable

in 11 of 13 control cattle. No disease was noted in any of the vaccinated cattle challenged at 3–13 weeks except two of three cattle receiving 1 ml (1/20) of V antigen with FCA.

Cattle produce a satisfactory immunity for three months after inoculation of white oil adjuvant vaccine, 50% protection at six months and no protection at nine months. The results indicated that the duration of immunity in cattle is less than six months. The white oil adjuvant vaccine was physically stable and produced good immunity after storage for four months at 4–8 °C.

In the field trial, 6310 cows in two provinces received white oil adjuvant vaccine. Only slight local swelling and a short transient fever was observed in a few animals. The vaccine appeared to be successful in reducing the prevalence of ephemeral fever in Shandong and Guandong Provinces as the incidence of disease in vaccinated herds was 7.8%, while in unvaccinated herds, the incidence reached 69%.

Neutralising antibodies in all vaccinated cattle were evaluated prior to the first vaccination and again on challenge. The results from pen trials are shown in Table 2. All cattle with neutralising antibody titres

Table 2. The relationship between post-vaccination serum neutralising antibody titre and challenge.

Animal no.	Vaccine volume (ml)	Vaccine dilution	Adjuvant	Antibody titre at challenge	Protection
87	2	1/1	FCA	128	+
88				77	+
89				256	+
99	1	1/1	FCA	64	+
100				64	+
90	2	1/20	FCA	< 4	+
91				11	+
92				< 4	+
93				< 4	+
94				16	+
95				< 4	+
117				16	+
118				16	+
96	1	1/20	FCA	< 4	−
97				5	+
98				< 4	−
114	1	1/1	WOA	16	+
115				16	+
116				16	+
119				16	+
120				16	+
111	2	1/20	WOA	16	+
112				16	+
113				16	+
121				16	+
122				16	+
123				16	+
124	2	1/40	WOA	16	+
125				16	+
126				16	+

FCA = Freunds complete adjuvant
WOA = White oil adjuvant

Analysis of the Antigens of Detergent Disrupted Bovine Ephemeral Fever Virus

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Abstract

In preliminary studies directed towards the development of a subunit vaccine, bovine ephemeral fever virus was disrupted by treatment with Triton X-100 and analysed for component proteins. Five proteins were detected in crude and purified preparations of disrupted virions.

THE earlier history of ephemeral fever vaccines in China has been described by Bai Wenbin (these proceedings). An inactivated vaccine prepared from BEF virus grown in suckling mouse brain provided good protection but had side effects which made it unsuitable for use as a vaccine. Cell culture adapted BEF virus which was inactivated with either beta-propiolactone or gentian violet and injected with aluminium hydroxide adjuvant provided only 50–80% protection. Since subunit vaccines have proven to be effective with other rhabdoviruses, research was undertaken on the development of an ephemeral fever subunit vaccine. The first stage in this research was to analyse the proteins derived from detergent disrupted virus.

Materials and Methods

The Beijing strain of BEF virus was used after passage through calves and adaption to BHK21 cell cultures. Detergent disrupted BEF virus was prepared by treating the virus pellet obtained by ultracentrifugation of cell culture medium with Triton X-100 followed by a second ultracentrifugation step.

A bovine antiserum to BEF virus was obtained by experimental infection of cattle with BEF virus. Monoclonal antibodies (DB5, 11A3 and 2C6) to BEF virus were obtained from CSIRO Australia. A rabbit anti-mouse immunoglobulin conjugated to horseradish peroxidase was obtained from the Beijing

Research Institute of Public Health. Horseradish peroxidase was conjugated to rabbit antibovine immunoglobulin.

Electrophoresis (SDS-PAGE) was carried out as described by Laemmli (1970) at a constant voltage of 200 V for 45 minutes at room temperature. The protein in the gel was transferred onto a nitrocellulose filter at a constant voltage of 30 V overnight in a buffer of 20% methanol 25 mM Tris pH 8.3 and 192 mM glycerine. The transferred nitrocellulose filter was blocked in 1% gelatin for one hour at room temperature and treated with BEF virus positive bovine serum or monoclonals, diluted in 0.05% Tween 20 in phosphate buffered saline (PBS), for one hour and then washed three times with 0.05% Tween 20 in PBS. Rabbit antimouse or antibovine horseradish conjugated immunoglobulin was added and incubated for one hour at room temperature, before washing four times. Finally, diaminobenzamine was used as an enzyme substrate to detect specific protein bands to which antibody had adhered.

Results and Discussion

When purified virus and the disrupted virus obtained by ultracentrifugation after treatment with Triton X-100, underwent electrophoresis and were stained with bromophenol blue, five structural proteins, designated L, G, N, M1 and M2 were observed. This result is similar to the observations of Walker et al. (1991).

The viral proteins were detected in both purified and crude preparation of disrupted virions. Immuno-

of >4 were resistant to challenge. Some cattle with a titre <4 were not protected while others with the same titre, were protected.

Discussion

Previous attempts to produce ephemeral fever vaccines involved either cell culture or mouse brain derived live or live-attenuated vaccines (Inaba et al. 1974; Vanselow et al. 1985; Erasmus 1986). However, our method is similar to the Triton X-100 treatment of infectious bovine rhinotracheitis (IBR) virus (Lupton and Reed 1980) and the NP-40 treatment of pseudorabies virus (Maes and Schutz 1983). Lupton and Reed (1980) reported that Triton X-100 did not destroy the biological activities or the immunogenicity of the envelope glycoprotein of IBR virus.

Our experiments demonstrated that a vaccine prepared from BEF virus cultures by ultracentrifugation and Triton X-100 solubilisation showed satisfactory immunogenicity in cattle. The results of the inoculation of V antigen into cell culture and laboratory animals indicates that BEF virus was completely disrupted by Triton X-100 treatment and had lost its infectivity. In experiments which compared the two adjuvants there was no apparent difference in immunogenicity but the white oil vaccine has advantages over Freunds complete adjuvant in toxicity and practicability. The use of even less toxic adjuvants such as Quil A needs to be investigated.

BEF virus neutralising antibody levels corresponded closely with immunity. Under our experimental conditions, all cattle which had an antibody titre of >4 were resistant to challenge. We consider that an antibody titre of >4 can be used as a marker of immunity although some cattle with a titre of <4 were resistant to challenge. It appears that humoral immunity plays an important role in immunity to BEF virus, but a cell-mediated response cannot be ruled out.

The duration of immunity was comparatively short. However, the strategic application of the vaccine, well before predicted ephemeral fever epidemics, can also produce satisfactory results. The experience in Shandong and Guangdong Provinces suggests that the vaccine can not only produce a good immunity in a healthy cattle herd, but also shorten the disease course during an endemic period.

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blotting of transferred proteins revealed that antibodies were particularly directed to glycoprotein, nucleoprotein and matrix protein. Monoclonals prepared in Australia to isolates from that country also reacted well with the proteins of Beijing strain. The highest molecular weight protein, protein L, could not be detected in immunoblotting, probably because of the difficulties involved in transferring it to nitrocellulose. Protein L is a viral polymerase considered to be unimportant in providing protective immunity against BEF virus infection. In contrast, protein G plays an important role in immunisation and was clearly demonstrated in immunoblots. It is clear that humoral immunity plays an important role in protection against BEF virus. Uren et al. (these proceedings) have shown the relative effects of G and N protein in lymphocyte stimulation assays but further research is necessary to elucidate their role.

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Epidemiological Investigations of Bovine Ephemeral Fever in Jilin Province

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Abstract

An epidemic of ephemeral fever occurred in four districts of Jilin Province in north-east China, between 10 August and 10 October 1991. Diagnosis was based on clinical signs and was confirmed by isolation of BEF virus and by serology. The number of cattle with clinical signs was 23 148 (10% morbidity) and the case fatality rate was 0.9%. The spread of the disease was not prevented by mountains or rivers, but within the affected areas there were foci where no cases occurred. This epidemic of ephemeral fever is the most northern (44 °N) to be reported in the northern hemisphere.

THE prevalence of bovine ephemeral fever in China has been previously reported (Bai Wenbin et al. 1991). Ephemeral fever usually occurs periodically, with intervals of 3–5 years, and causes severe economic loss for the cattle industry. In August 1991 a disease with clinical signs indistinguishable from ephemeral fever occured in cattle in Jilin municipality and in Yanbian district of Jilin Province. It appeared at the same time in 4–5 counties, and caused severe economic loss for the cattle industry and agricultural production. This paper reports the epidemiology, pathogen identification, serum antibody detection, preventive measures and treatment of cattle involved in the 1991 Jilin epidemic.

Epidemiology

The disease began in some districts in Jilin Province in Northern China in August 1991, with a very rapid spread. Within 10 days, the disease was reported in several counties. Intensive investigations were carried out in order to understand the epidemiology of the disease, the results are presented in Tables 1 and 2. The total number of exposed animals was 232 822 of which 23 418 showed evidence of clinical signs (morbidity rate 10%) and 204 of these animals died (mortality rate 0.9%).

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In Hunjiang district, the disease was first reported on 10 August 1991 and the final case on 10 October 1991, a duration of 60 days. In Jilin municipality and Tonghua district, the outbreak lasted 40 days, while in Yanbian district the duration was 30 days. In Huadian county of Jilin municipality and Dunhua city of Yanbian district, the disease began in August but stopped abruptly after only 7–15 days.

The spread of the disease was very rapid and was not stopped by mountains and rivers. Areas of disease activity were usually not connected to each other to form a single large area. Within the endemic areas there were also foci where the disease did not occur.

Clinical signs observed were similar in cattle throughout Jilin Province. The first obvious sign was the sudden onset of fever with body temperature rising to 41–42.5 °C for 2–3 days. In most cases the respiratory rate was accelerated and nasal discharge was common. Anorexia and rumen stasis often developed and there was mucus in the faeces. The diseased animals showed muscular shivering and stiffness as well as swollen joints in all four legs, which resulted in great difficulty in movement, or even paralysis in some cases. The milk yield dropped sharply and in some cases there was blood in the milk. Abortion was a frequent finding after the fever had subsided. To investigate the relationship between age and disease, the records for Jiaohe county were examined. Of 207 cattle which showed clinical signs of disease, 77% were in the 6–8 year age group (Table 2).

Table 1. Morbidity and mortality data for the 1991 epidemic of ephemeral fever in Jilin Province.

Location	Date of occurrence	Total number of cattle	Number of diseased cattle	Morbidity (%)	Number of dead cattle	Mortality (%)
Jilin municipality	20 Aug–1 Oct	17 487	1 688	10	24	1.4
Yanbian district	1 Sept–30 Sept	17 614	1 646	9	4	0.2
Hunjiang district	10 Aug–10 Oct	54 505	15 744	29	157	1.1
Tonghua district	20 Aug–30 Sept	143 216	4 340	3	19	0.4
Totals	10 Aug–10 Oct	232 822	23 418	10	204	0.9

Table 2. The relationship between animal age and morbidity in cattle in the 1991 epidemic of ephemeral fever in Jiaohe County.

Animal age (years)	3	4	5	6	7	8	9	11
Number diseased	6	6	15	36	60	63	20	1
Morbidity (%)	3	3	7	17	29	30.3	10	0.5

Virus Isolation and Serology

The clinical diagnosis of ephemeral fever was confirmed by virus isolation and by serology on paired sera collected during acute and convalescent stages from cattle in Huadian county in Jilin municipality. For virus isolation, citrated blood collected from animals with fever, was centrifuged and the leucocyte layer collected and inoculated onto C6/36 (*Aedes albopictus*) cell cultures. The cell cultures were incubated for 12–14 hours then fixed with acetone and air dried. The fixed cells were incubated with murine anti-BEF virus antiserum for 30 minutes, washed and then incubated for 30 minmutes with a rabbit antimurine immunofluorescent antibody conjugate. After a washing step the slides were examined with a fluorescence microscope. This procedure identifies viruses of the BEF virus serogroup but does not distinguish among them. Viruses which were isolated in C6/36 cells were transferred to BHK21 cell cultures for another one or two passages. Cell culture fluids from cultures with obvious cytopathic effects were negatively stained and examined for typical rhabdovirus morphology using an electron microscope.

For detection of serum neutralising antibody to BEF virus, seven serum samples from convalescent cases, one sample from a clinically normal but in-contact animal and two samples from healthy cattle from an area where ephemeral fever had not been reported, were used in cell culture and suckling mice neutralisation tests. Antibody was detected in sera from convalescent cases but not in samples obtained from clinically unaffected animals.

Prevention and Treatment

During the epidemics, quarantine was imposed and animal markets were closed. Diseased cattle were separated and not allowed to move to other cattle pens or villages. Cattle pens as well as cattle were cleaned and sprayed with disinfectants each day in order to maintain hygiene, kill insects and minimise disease transmission. Medical treatment was carried out according to the clinical signs observed. Cattle with fever and hypomotile rumen were treated with antipyretics, analgesics and anodynes. Cattle with lameness were treated with salicylic acid while cattle which may have had secondary infection and had more severe respiratory signs were treated with penicillin and streptomycin. Apart from the above treatments, intravenous infusion of hydrogen peroxide was given to cattle with emphysema. Paralysed cattle were treated with strychnine. In addition, diseased animals were carefully looked after and given easily digested food and vitamins to speed recovery.

Discussion

Isolation and identification of BEF virus and specific serological examination demonstrated that the disease prevalent in cattle in Jilin Province in August,

1991 was ephemeral fever. Epidemiological investigations in Jilin province showed that the disease was present in many locations for two months, although in some areas the disease was present for only 7-15 days.

Inaba (1973) reported that ephemeral fever in Japan usually occurred in late summer or early autumn and that the disease had not occured north of 38 °N lattitude. Standfast et al. (1973) reported that ephemeral fever endemic areas in Australia were the southern hemisphere equivalent of those in Japan. However, in the late summer and early autumn of 1991, ephemeral fever occured in Jilin Province, at 44 °N lattitude, very different from the limits reported in Japan and Australia.

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Epidemiology and Prevention of Bovine Ephemeral Fever in Guangdong Province

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Abstract

Ephemeral fever was first reported in Guangdong Province in 1955. Small scale outbreaks have recurred at approximately two year intervals since then and a large scale outbreak has occurred approximately every four years. Since 1990, when better records were kept, ephemeral fever has been diagnosed every month in Guangdong Province. BEF virus was isolated in 1977 and was designated the Huixing 771214 strain. This virus was used as a mouse brain grown vaccine to vaccinate 5000 cattle in 1978-1979. The vaccine provided full protection after three doses but side effects prevented its further use. A live virus vaccine based on the Beijing 1 strain gave inadequate protection as did an Australian live virus vaccine. A subunit vaccine, supplied by the Harbin Veterinary Research Institute, was used to vaccinate dairy cattle prior to the 1991 epidemic and appeared to reduce the number of cases of disease and to reduce the duration of the epidemic to 10-15 days on individual farms.

EPHEMERAL fever was first reported in Guangdong in 1955. During 1962-1977, in Xingshou farm, Guangzhou, ephemeral fever was recorded six times, involving a total of 4028 cattle. The average morbidity was 20% and the average mortality was 4%. The morbidity rate was higher in young cattle (34%), while in adult cattle the rate was 16%, and in calves, 5%. Records for the occurrence of the disease on dairy farms in 1983, 1987 and 1990 are shown in Table 1.

Table 1. The morbidity and mortality rates in dairy cattle in Guangzou Municipality, Guangdong Province.

Year	Total No. of cattle	Morbidity	Mortality
1983	7 831	3 124 (40%)	141 (4%)
1987	11 145	3 222 (28%)	121 (4%)
1990	17 396	282 (1.5%)	9 (3%)

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The prevalence of ephemeral fever varies from year to year. Records indicate that small scale outbreaks, involving only a small number of animals, occur about every second year, with a large scale outbreak occurring about every fourth year. However the situation is very variable and sometimes ephemeral fever appears to occur continuously over a two or four year period. Since 1990, when better records of ephemeral fever were kept, the disease has been diagnosed in every month of the year in Guangdong Province. The calculated economic loss caused by ephemeral fever has been significant. Although the records are incomplete, losses in 1977 were calculated to be RMB 200 000, while in 1990 the loss was calculated to be RMB 800 000 and RMB 240 000 in 1991.

Before 1977, ephemeral fever in Guangdong Province was often misnamed as bovine influenza. In that year, BEF virus was isolated, in suckling mouse brain, from blood collected from an animal with an elevated temperature. This isolate, designated Huixing 771214, was shown to be BEF virus by cross neutralisation tests with the Beijing 76AMH isolate (see Liu Shanggao, these proceedings).

In 1978 and 1979, research was undertaken to develop a mouse brain vaccine based on the Huixing 771214 isolate. The preparation was used to vaccinate 5000 cattle, some of which were experimentally

challenged with virulent virus. After one dose of vaccine, 80% of recipients were protected against challenge. Vaccination of cattle with two doses of vaccine increased the protection rate to 90%, while three doses provided 100% protection. In spite of the excellent protection achieved, this work was not continued because of serious side effects which resulted from the mouse brain component of the vaccine. In 1987, 107 cows in Yantang farm were vaccinated with a live cell culture derived vaccine made from the Beijing 1 strain by the staff of Harbin Veterinary Research Institute, but satisfactory protection against disease was not achieved. In 1987, an Australian commercial vaccine was used to vaccinate 754 cows in Zhuchen farm where an outbreak had just begun. In spite of the use of the vaccine, 122 (16%) animals still developed the disease during an epidemic.

In 1988, a subunit vaccine developed by Harbin Veterinary Research Institute (see Bai Wenbin et al. these proceedings) was field tested in Guangdong Province. The vaccine was first used in Yantang farm for a two-year period. In 1990, the use of the vaccine was expanded to include other parts of Guangzhou

and a total of 6124 cattle were vaccinated. The vaccine was considered to be safe since no clinical signs were associated with its use, apart from a localised reaction at the injection site. The reaction was much reduced by changing from subcutaneous to intramuscular injections. Although detailed records are not available, the number of cattle showing clinical signs of ephemeral fever appeared to be lower than on non-vaccinated farms. The duration of the outbreak was apparently also shorter on the vaccinated farms.

Based on previous observations of ephemeral fever in Guangdong Province, it was predicted that a major epidemic would occur in 1991. Consequently, vaccination was carried out on most cattle farms in Guangdong province well before the predicted outbreak. When clinical cases did begin to appear, the epidemic ceased in 10 to 15 days. Experience from the use of the subunit vaccine in 1990-91, indicated that calves should be vaccinated early and young cattle should be vaccinated twice. To fully prepare for future outbreaks of ephemeral fever, an increase in serological monitoring would be helpful towards understanding the epidemiology of the disease and to assist in the prediction of outbreaks.

Antibody and Cell Proliferative Responses of Cattle Vaccinated with Bovine Ephemeral Fever Virus Proteins

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Abstract

Three groups of cattle were vaccinated on two occassions, 14 days apart, with purified proteins derived from BEF virus and were then challenged with virulent virus. A control group served as unvaccinated controls. Group 1 received two doses of glycoprotein (G), group 2 received two doses of nucleoprotein (N) and group 3 received received an initial dose of glycoprotein followed by a dose of nucleoprotein (G + N). Only group 1 was protected against against challenge. T lymphocyte proliferation in response to BEF virus was observed in cattle vaccinated with either glycoprotein or nucleoprotein, indicating the presence of G and N specific T cells.

BOVINE ephemeral fever (BEF) virus is an arthropod-borne rhabdovirus of the genus *Lyssavirus* (Calisher et al. 1989). The virion consists of five structural proteins (L, G, N, M1 and M2) which correspond with other members of the genus of which rabies virus and vesicular stomatitis virus (VSV) are the most studied (Walker et al. 1991). The ongoing development of effective subunit vaccines against rabies virus, VSV and BEF virus has been based on the use of individual or combinations of viral proteins as immunogens. The glycosylated envelope (G) proteins of these three viruses have been shown to be responsible for stimulating protective virus neutralising antibodies in a variety of species (Wunner at al. 1983; Mackett et al. 1985; M.F. Uren unpublished data). The rabies virus and VSV nucleoproteins (N) are also capable of inducing an immune response, but this response is variable and may not be fully protective (Fu et al. 1991; Fekadu et al. 1992). Both the G and N proteins of rabies and VSV also produce T cell proliferative responses (Puddington et al. 1986; Macfarlan et al. 1984; Fu et al. 1991). This paper describes the T cell proliferative and specific antibody response of cattle to vaccination with the G and N proteins of BEF virus.

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Material and Methods

Virus

The Australian prototype BB7721 strain of BEF virus (Doherty et al. 1969) was used in all procedures. The challenge virus for cattle inoculation was also derived from the same clinically ill animal but this virus had been maintained by serial passage in cattle. The G and N proteins of BEF virus were isolated by size exclusion HPLC (see Riding et al. these proceedings) from gradient purified BEF virus grown in BHK21 cells (Walker et al. 1991).

Vaccination schedule

Twelve, 18-month old crossbred dairy cattle, free from BEF virus neutralising antibody, were divided into four groups of three animals as shown in Table 1. The adjuvant Quil A (Superfos Biosector a/s, Denmark) was used in all vaccine preparations at a concentration of 1.0 mg/ml. Serum for serological testing was collected at 14-day intervals. EDTA-treated blood was collected from all cattle 30 days after the second vaccination for lymphocyte proliferation assays. All cattle were challenged by intravenous inoculation of 2.0 ml of BEF virus infected cattle blood 40 days after the second vaccination. After challenge all animals were monitored daily for clinical signs and blood collected for virus isolation.

Table 1. Vaccination schedule.

Group	First vaccination — day 0		Second vaccination — day 21	
	Protein	Dose μg/dose	Protein	Dose μg/dose
Group 1 (G + G)	G	1.0	G	1.0
Group 2 (N + N)	N	10.0	N	10.0
Group 3 (G + N)	G	1.0	N	10.0
Control	—	0	—	0

Serology

Microtitre serum neutralisation tests were carried out by the method of Cybinski and Zakrzewski (1983). A blocking ELISA for the G protein of BEF virus was conducted by the method described by Zakrzewski et al. (1992). A blocking ELISA for the N protein of BEF virus used the same method but substituted an N protein specific monoclonal antibody as the detecting antibody and N protein as the coating antigen.

Lymphocyte proliferation assay

The stock medium used in all lymphocyte proliferation studies was Dulbecco's modified Eagle medium (GIBCO, USA) supplemented with glucose (4.0 g/l), folic acid (6.0 mg/l), L-arginine (116.0 mg/l), L-asparagine (36.0 mg/l), HEPES (2.383 g/l) and sodium bicarbonate (2.0 g/l). Before use the medium was also supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol and 200 units/ml of penicillin/ streptomycin. This medium was termed DMEM plus.

Bovine lymphocytes were isolated from EDTA-treated bovine peripheral blood by the cold water lysis method described by Riding and Willadsen (1981). The lymphocytes were resuspended in DMEM plus at a cell concentration of 10×10^6 per ml. Fifty microlitre aliquots of cell suspension were then dispensed in triplicate into 96 well plates, followed by 150 μl of fresh medium. Lymphocytes were stimulated by the addition of $10^{7.45}$ TCID$_{50}$ of purified BEF virus to each well, while unstimulated wells were included as controls. Plates were then incubated at 37 °C in a 4% CO$_2$ incubator. A kinetic analysis was conducted with plates harvested at days 4, 6, 8, 10 and 12 post-stimulation. Cell proliferation was determined by [methyl-^{3}H] thymidine (Amersham, England) uptake (0.625 μCi per well) over the final 24-hour pulse period.

Results

The only cattle that were protected from challenge with virulent BEF virus were in group 1(G + G). Those in groups 2(N + N), 3(G + N) and controls, all exhibited typical clinical signs of ephemeral fever, including pyrexia, lameness, anorexia, nasal and ocular discharges. During the acute phase, BEF virus was isolated only from groups N + N, G + N and the controls (data not shown).

The cell proliferation assay results are shown in Figure 1. There was a cell proliferative response by cattle in groups 1 and 2, which had been vaccinated twice with either G or N proteins. There was no specific response in group 3(G + N) or the control group.

The results of the virus neutralisation test (Figure 2) show that neutralising antibody was produced by group 1 after vaccination. Group 3 produced low levels of neutralising antibody (titre < 4); the remaining two groups did not produce neutralising antibodies to BEF virus. The results of the G and N protein blocking ELISA are shown in Figures 3a and 3b. The G protein blocking ELISA results show that group 1 had a rise in inhibiting antibody after vaccination (> 60% inhibition), with group 3 showing a lower response (< 50% inhibition). The remaining groups did not show a response until after challenge (data not shown). The N protein blocking ELISA results show a rise in inhibiting antibody after vaccination in group 2 and no significant response in group 3.

Discussion

In natural cases of ephemeral fever, the immunity arising from a single infection usually gives lifelong protection (St George 1981). If a vaccination regime were to mimic this durable immunity it may be
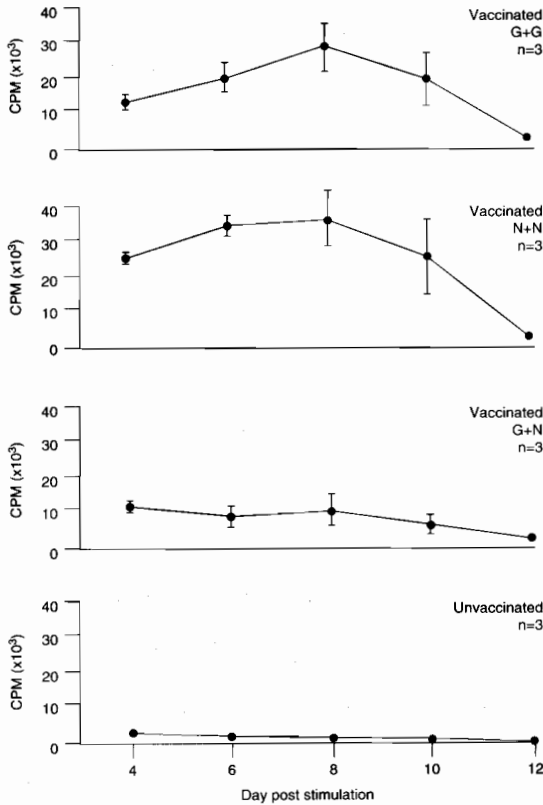
Fig. 1. Lymphocyte proliferative response to *in vitro* stimulation with BEF virus of cattle vaccinated against different BEF viral proteins. Group 1 (G + G) were vaccinated with G protein on days 0 and 21. Group 2 (N + N) were vaccinated with N protein on days 0 and 21. Group 3 (G + N) were vaccinated with G protein on day 0 and N protein on day 21. The T cells were stimulated *in vitro* on day 51. The results are shown as cpm × 10³ ± S.E.

Fig. 2. BEF virus neutralising antibody response of cattle following vaccination with different BEF viral proteins and subsequent challenge with BEF virus. Group 1 (G + G) were vaccinated with G protein on days 0 and 21. Group 2 (N + N) were vaccinated with N protein on days 0 and 21. Group 3 (G + N) were vaccinated with G protein on day 0 and N protein on day 21. All cattle were challenged on day 61. The results are expressed as the $\log_{10}$ of the neutralising titre ± S.E.

necessary to incorporate more than one protein in a sub-unit vaccine to stimulate a more complete repertoire of T cell and antibody responses. The results of this study have shown that the vaccination of cattle with G and N proteins can stimulate a T lymphocyte response to BEF virus. The proliferation assay result for group 3 demonstrates that the different T lymphocyte epitopes on the G and N proteins that are responsible for this response are not cross-reactive. This study has also established that vaccination with BEF virus G protein stimulates the production of G protein-specific neutralising antibodies that protect cattle from challenge, while vaccination with BEF virus N protein produces N protein-specific non-neutralising antibodies that did not confer protection.
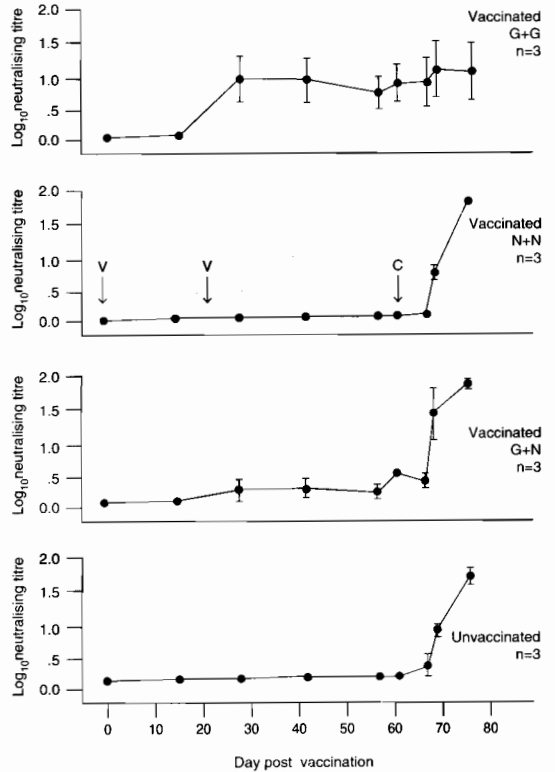
Fekadu et al. (1992) have shown that vaccination of dogs with rabies virus G protein produced virus neutralising antibodies and conferred full protection, while vaccination with N protein did not produce virus neutralising antibodies but did offer partial protection. Fu et al. (1991) reported that immunisation of mice with rabies virus N protein gave full protection. In our study there was no protection of cattle vaccinated with N protein of BEF virus, although there was a T cell response. The difference in these responses between BEF virus and rabies virus may be attributable to different modes of infection or sites of replication. Alternatively, the much longer incubation time and therefore exposure to rabies virus antigens may have some influence.
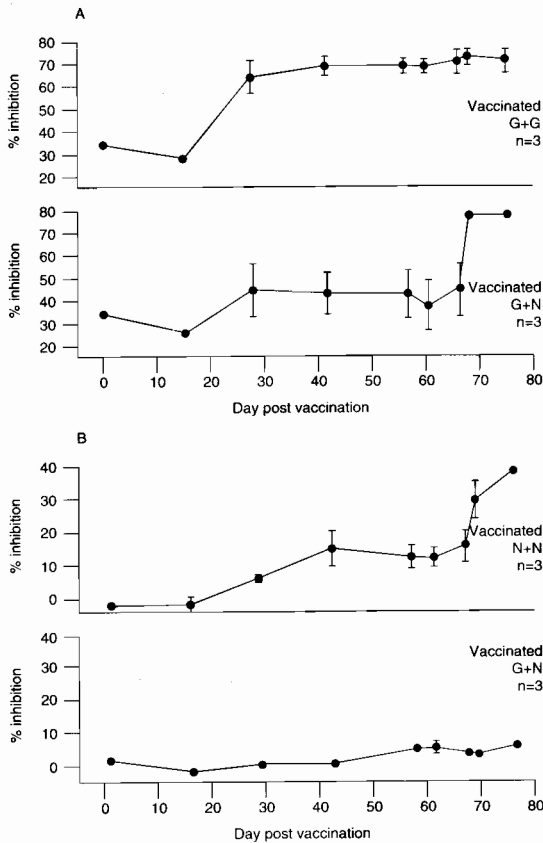
Fig. 3. BEF virus protein specific blocking ELISA antibody response of cattle following vaccination with different BEF viral proteins and subsequent challenge with BEF virus. Group 1 (G + G) were vaccinated with G protein on days 0 and 21. Group 2 (N + N) were vaccinated with N protein on days 0 and 21. Group 3 (G + N) were vaccinated with G protein on day 0 and N protein on day 21. All cattle were challenged on day 61. The results are expressed as the % inhibition ± S.E. **(A)** BEF virus G protein specific blocking ELISA. **(B)** BEF virus N protein specific ELISA.

The correlation between protection and BEF virus G protein-produced neutralising antibodies confirms the work of Cybinski et al. (1990) who used passive immunisation of mice as a model. The low level G protein antibodies produced by group 3 (G + N) were not protective and this confirms a previous observation (data not shown) that > 60% inhibition is required in a BEF virus, G protein blocking ELISA to protect against infection.

It has been established that in many viral infections a strong T cell response enhances the subsequent antibody response upon challenge (Francis et al. 1987; Ertl et al. 1989; Roehrig et al. 1992).

Previous studies have shown that vaccination of cattle with two doses of BEF virus G protein produces effective short-term immunity for 9–12 months (see Bai Wenbin et al. these proceedings). This limited immunity may be due to the lack of adequate T cell epitopes necessary to produce a strong and durable antibody response. An ephemeral fever vaccine combining both the G protein and the N protein may offer better long-term protection by enhancement of the antibody response via lymphokine release from T cells stimulated by both G and N proteins.

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Bovine Ephemeral Fever Strain Variation

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Abstract

Isolates of bovine ephemeral fever virus from different topographical locations during different epidemics in Australia were compared using a panel of monoclonal antibodies. All isolates were neutralised by monoclonals 17B1, 13A3, 15B5, 12A5, 16A6, 5A5 and a polyclonal mouse ascitic fluid antiserum. Major differences between isolates occurred in the 8B6-defined epitope in antigenic site G3a on the major glycosylated envelope protein which underwent a change in 1973–1974. The 8D3-defined epitope in antigenic site G3b varied with different isolates but did not show any correlation with time, site or host. Variations in BEF virus isolates, as defined by monoclonal antibodies, may influence the development of effective vaccines.

EPHEMERAL fever is a disease of cattle and water buffalo which occurs in Africa, Asia and Australia (St George 1981). BEF virus is an arthropod-borne virus of the family Rhabdoviridae, genus *Lyssavirus* (Calisher et al. 1989). Until recently, studies have shown no significant variation among isolates of BEF virus from within Australia or from different countries (Inaba et al. 1969; Snowdon 1970; Tian et al. 1987). These studies utilised polyclonal antibodies produced in a variety of laboratory animals. The recent development of monoclonal antibodies against BEF virus by Cybinski et al. (1990, 1992) has allowed a more detailed analysis of the antigenic structure of BEF virus. Five distinct antigenic sites (G1, G2, G3a, G3b and G4) on the glycosylated envelope (G) protein have been identified. Cybinski et al. (1992) assayed 18 isolates of BEF virus from Australia using three monoclonal antibodies specific to the G protein of BEF virus. They found that BEF virus strains could be differentiated by the presence or absence of a monoclonal antibody defined epitope in antigenic site G3a. This paper describes a continuation of those studies, with 70 isolates of BEF virus from Australia being asssayed against nine monoclonal antibodies specific to the G protein of BEF virus.

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Materials and Methods

BEF virus isolates listed by identifying isolate number, site of collection and year of collection are listed in Table 1. The eight virus neutralising and one non-neutralising monoclonal antibodies used in this study were fully described by Cybinski et al. (1990, 1992). Monoclonals 17B1 and 13A3 are specific for antigenic site G1; 15B5 and 12A5 for antigenic site G2; 8B6 for antigenic site G3a; 8D3 and 16A6 for antigenic site G3b; 5A5 for antigenic site G4; and 3A2 is non-neutralising G protein specific. A polyclonal mouse ascitic fluid antiserum against BEF virus was also used as a control. The viruses were assayed using an immunofluoresence inhibition assay described by Cybinski et al. (1992).

Results and Discussion

All isolates were neutralised by monoclonal antibodies 17B1, 13A3, 15B5, 12A5, 16A6, 5A5 and the polyclonal mouse ascitic fluid antiserum. One monoclonal antibody, 3A2, neutralised one isolate (CSIRO1867), but none of the other isolates including the prototype strain BB7721. The other two monoclonal antibodies (8B6 and 8D3) gave variable patterns of neutralisation and these are listed in Table 1. The results demonstrate that the 8B6-defined epitope is generally absent on BEF virus isolates after 1973–1974. The 8D3-defined epitope gave an erratic result with no definite pattern.

Table 1. Australian BEF virus isolates tested for neutralisation by monoclonal antibodies using immunofluorescence inhibition.

Virus isolate	Location	Year	Monoclonal antibody (antigenic site)		
			6.8B6 (G3a)	5.8D3 (G3b)	6.3A2 (G Nn[1])
V1956[2]	Sydney, NSW	1956	+	+	−
BB7721	Charters Towers, Q	1968	+	+	−
CSIRO 1865	Charters Towers, Q	1968	+	−	−
CSIRO 1818	Upper Barron, Q	1970	+	−	−
CSIRO 660	Etna Creek, Q	1970	+	+	−
CSIRO 1867	Etna Creek, Q	1970	+	+	+
CSIRO 1869	Etna Creek, Q	1970	+	+	−
CSIRO 1870	Etna Creek, Q	1970	+	+	−
CSIRO 1873	Etna Creek, Q	1970	+	+	−
CSIRO 1902	Etna Creek, Q	1970	−	+	−
CSIRO 1903	Etna Creek, Q	1970	−	−	−
CSIRO 1904	Etna Creek, Q	1970	+	+	−
CSIRO 1938	Munna, Q	1970	+	+	−
CSIRO 1939	Etna Creek, Q	1970	+	−	−
CSIRO 1940	Etna Creek, Q	1970	+	+	−
CSIRO 1942	Camp Mountain, Q	1971	+	+	−
CSIRO 1943	Yeerongpilly, Q	1972	+	+	−
CSIRO 1819	Wacol, Q	1973	+	+	−
CSIRO 1930	Etna Creek, Q	1973	+	−	−
CSIRO 1931	Etna Creek, Q	1973	+	+	−
CSIRO 1932	Etna Creek, Q	1973	+	−	−
CSIRO 1933	Etna Creek, Q	1973	+	−	−
CSIRO 1935	Belmont, Q	1973	−	+	−
V393	Scone, NSW	1973	−	−	−
CSIRO53[3]	Etna Creek, Q	1974	+	+	−
V496	NSW	1974	−	+	−
V511	NSW	1974	−	+	−
CSIRO 42[4]	Beatrice Hill, NT	1975	+	−	−
CSIRO 1821	Amberley, Q	1975	−	+	−
CSIRO 1925	Tolga, Q	1975	−	+	−
CSIRO 1926	Kairi, Q	1975	−	+	−
CSIRO 1927	Kairi, Q	1975	−	+	−
CSIRO 1820	Peachester, Q	1976	−	+	−
CSIRO 968	Jimboomba, Q	1979	−	+	−
CSIRO 355	Peachester, Q	1980	−	+	−
CSIRO 366	Peachester, Q	1980	±	−	−
DPP54	Tortilla Flats, Q	1981	+	±	−
CSIRO 1362	Samford, Q	1981	−	+	−
CSIRO 967	Kairi, Q	1981	−	+	−
CSIRO 1179	Peachester, Q	1982	−	+	−
CSIRO 1180	Peachester, Q	1982	−	+	−
CSIRO 1188	Long Pocket, Q	1982	−	+	−
CSIRO 1204	Jimboomba, Q	1982	−	+	−
CSIRO 1618	Peachester, Q	1984	−	+	−
CSIRO 1619	Peachester, Q	1984	−	+	−
CSIRO 1622[4]	Peachester, Q	1984	−	+	−
CSIRO 1647[5]	Peachester, Q	1984	−	+	−
CSIRO 1899	Boonah, Q	1988	−	+	−
CSIRO 1900	Peachester, Q	1988	−	+	−
CSIRO 1901	Peachester, Q	1988	−	+	−
CSIRO 1905	Amberley, Q	1989	−	+	−
CSIRO 1906	Samford, Q	1989	−	+	−
CSIRO 1907	Samford, Q	1989	−	+	−

Table 1. (continued)

Virus isolate	Location	Year	Monoclonal antibody (antigenic site)		
			6.8B6 (G3a)	5.8D3 (G3b)	6.3A2 (G Nn[1])
CSIRO 1908	Samford, Q	1989	−	+	−
CSIRO 1910	Samford, Q	1989	−	+	−
CSIRO 1911	Closeburn, Q	1989	−	±	−
CSIRO 1912	Samford, Q	1989	−	±	−
CSIRO 1913	Oakey, Q	1989	±	+	−
CSIRO 1914	Toowoomba, Q	1989	−	±	−
CSIRO 1915	Amberley, Q	1989	−	−	−
CSIRO 1917	Oakey, Q	1989	−	+	−
CSIRO 1918	Oakey, Q	1989	−	+	−
CSIRO 1920	Injune, Q	1989	−	+	−
CSIRO 1921	Injune, Q	1989	−	±	−
CSIRO 1922	Cunnamulla, Q	1989	+	−	−
CSIRO 1923	Cunnamulla, Q	1989	−	−	−
CSIRO 1924	Roma, Q	1989	−	−	−
CSIRO 1947	Calliope, Q	1989	−	−	−
CSIRO 1951	Willowbank, Q	1992	−	+	−
CSIRO 1952	Amberley, Q	1992	−	+	−

[1] Nn = non-neutralising
[2] Blood collected from a fertile animal and stored for several years before being processed for virus isolation
[3] *Culicine mosquitoes*
[4] *Anopheles bancrofti*
[5] *Culicoides brevitarsis*
+ Neutralization
− No neutralization
± Equivocal result
Note: NSW = New South Wales; Q = Queensland; NT = Northern Territory.
 All strains were isolated from cattle unless marked.

In this study the virus isolates were chosen to cover a comprehensive selection of BEF viruses isolated in Australia. They represent isolates from all major epidemics since 1956 (T.D. St George pers. comm.). Within individual epidemics, a selection was made of multiple isolates from the same site and multiple isolates from multiple sites (Table 1). Isolates were also selected from the same site over consecutive epidemics, for example, samples from Peachester in southeast Queensland for years 1976, 1982, 1984 and 1988.

The major differences were detected in antigenic site G3, although there was also a single variation in the 3A2-defined non-neutralising epitope. The 8B6-defined epitope in antigenic site G3a appeared to undergo a change in 1973–1974. In the period 1956–1973, 21 of 26 isolates (81%) possessed the 8B6 epitope but from 1974–1992, only 4 of 44 isolates (9%) reacted with 8B6. This result corresponds with our previous report that the 3D6-defined epitope, in site G3a, also changed in the same time-period (Cybinski et al. 1992). The 8D3-defined epitope in

antigenic site G3b varied with different isolates but did not show any correlation with time, site or host. Whether this reflects a loss of neutralising ability for this epitope or a fluctuation in neutralising titre is not known at this stage. In contrast, the other epitope in antigenic site G3b, 16A6, did not exhibit any variation. The epitopes in antigenic sites G1, G2 and G4 also did not show any change.

These changes in the BEF virus G protein have not been reflected in any change to the disease characteristics (T.D. St George and M.F. Uren, pers. comm.).

Flamand et al. (1980) have described monoclonal antibody defined variation in the glycoprotein of rabies virus, a related *Lyssavirus*. Dietzschold et al. (1988) have demonstrated that rabies virus isolates from different origins exhibit significant variation and that these variations have important implications for vaccine development.

In Australia and China, the BEF viruses used in vaccine development have been the initial isolation

in each country, isolated in 1968 and 1976 respectively (Vanselow et al. 1985; Bai et al. these proceedings). Our results now show that in Australia, the BEF viruses circulating since 1973–74 are different in at least one antigenic site to the vaccine strain. The results in this study confirm our earlier observations (Cybinski et al. 1992), and indicate that variation should be taken into consideration when developing vaccines based on individual virus proteins. A program of regular monitoring of BEF viruses circulating in the cattle population would assist in identifying further changes and ensure the continuing effectiveness of any vaccine.

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Antigenic Variation in the Bovine Ephemeral Fever Virus Glycoprotein

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Abstract

Antigenic variation in the bovine ephemeral fever virus glycoprotein was demonstrated using monoclonal antibodies and comparing a particular passage level of the BB7721 strain with (1) other Australian isolates of BEF virus, (2) the Beijing 1 strain of BEF virus, isolated in China and (3) batches of the BB7721 strain with different passage histories. Escape mutants of BEF virus were selected from cell culture and suckling mice, by growing virus in the presence of neutralising monoclonal antibodies. Escape frequencies were calculated for 14 monoclonal antibodies and an epitope map constructed of antigenic sites on the BEF viral glycoprotein which induce the production of neutralising antibodies.

RABIES viruses isolated from different animal species in various parts of the world were formerly considered to be closely related (Wiktor and Clarke 1973). However, more recent studies using monoclonal antibodies against the nucleocapsid protein and the glycoprotein of the virus have provided evidence of antigenic differences among several strains of rabies virus (Wiktor and Koprowski 1980). It has been suggested that these differences might have been responsible for occasional failures in postexposure vaccination of rabies patients. Furthermore, when mice were vaccinated with standard rabies vaccine and then challenged with street viruses isolated from fatal cases of human rabies, some instances of vaccine failure were observed (Wiktor and Koprowski 1980). Considerable genetic diversity has also been demonstrated for strains of vesicular stomatitis virus, using T1 ribonuclease fingerprinting (Nichol 1988). Using monoclonal antibodies (MAbs), it has now been shown that variation also exists in the bovine ephemeral fever (BEF) virus glycoprotein (Cybinski et al. 1990, 1992).

Materials, Methods and Results

Viruses

The Australian virus isolates which were compared to the BB7721 strain of BEF virus included the insect isolates CSIRO 42 and CSIRO 53 viruses (Standfast et al. 1984) and the closely related Berrimah virus (Gard et al. 1983). The Beijing 1 strain of BEF virus from China (Zhai et al. 1980) was also compared. CSIRO 42 virus was isolated from *Anopheles bancrofti* mosquitoes collected at Beatrice Hill in the Northern Territory. CSIRO 53 virus was isolated from a mixed mosquito pool of Culicine mosquitoes collected at Etna Creek near Rockhampton in Queensland. Berrimah virus was isolated from the blood of a healthy steer located in the north of the Northern Territory. Neutralisation testing carried out on this animal for two years revealed high levels of neutralising antibody to both Berrimah and BEF viruses prior to the isolation of Berrimah virus (Cybinski 1987). This indicated a previous infection with at least one and probably several BEF-related viruses (Figure 1). The Beijing 1 strain of BEF virus was isolated in China from bovine blood.

Cross-reactivity of BEF virus isolates using polyclonal antibodies

Cross neutralisation tests on the Australian isolates, using polyclonal antibodies indicated some minor differences among the BB7721, CSIRO 42 and CSIRO 53 viruses, while Berrimah virus was considered to be a different virus, although closely related (Table 1). Testing has not been completed on the Beijing 1 virus, although the results so far, using polyclonal antibodies, indicate that there is little difference between the Chinese and Australian viruses.

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Table 2. Viral specificity of neutralising monoclonal antibodies by neutralisation and immunofluorescence to isolates of BEF virus.

G protein monoclonal antibody	Neutralisation titres[1] of ascitic fluids (immunofluorescence reaction)			
	BEF (BB7721)	BEF (CSIRO42)	BEF (CSIRO53)	BER (DPP63)
DB5	512(+)	256(+)	256(+)	<2(−)
13C6	4096(+)	2048(+)	2048(+)	<2(+)
13A3	1024(+)	3072(+)	1024(+)	<2(−)
17B1	1024(+)	2048(+)	1024(+)	<2(+)
9C5	128(+)	384(+)	128(+)	96(+)
1C6	256(+)	256(+)	384(+)	128(+)
12A5	256(+)	512(+)	512(+)	24(+)
15B5	512(+)	256(+)	512(+)	128(+)
11D1	4(+)	16(+)	8(+)	8(+)
3D6	512(+)	256(+)	<2(−)	<2(+)
8B6	16(+)	6(−)	<2(−)	<2(−)
16A6	8(+)	16(+)	8(+)	8(+)
8D3	8(+)	<2(+)	8(+)	8(+)
5A5	512(+)	32(+)	128(+)	16(+)

[1] Reciprocal of antibody dilution which neutralised 100 TCID$_{50}$ of virus in 50% of the wells
+ = immunofluorescence   − = no immunofluorescence

Table 3. Viral specificity (indirect immunofluorescence) of non-neutralising monoclonal antibodies to isolates of BEF virus.

Monoclonal antibody	BEF protein specificity	Immunofluorescence reactions of hybridoma supernatants			
		BEF (BB7721)	BEF (CSIRO42)	BEF (CSIRO53)	BER (DPP63)
18D2	G	+	−	−	−
3A2	G	+	+	+	+
11B5	G	+	+	−	−
10B1	G	+	+	+	+
13B5	G	+	+	+	−
2C4	G	+	−	−	−
18C2	G	+	+	−	−
EB4	G	+	+	+	−
2C6	M2	+	+	+	+
FD2	M2	+	+	+	+
4A4	M2	+	+	+	+
17A3	M2	+	+	+	+
20A6	M2	+	+	+	+
11A3	N	+	+	+	+
6C1	N	+	+	+	+
11B5	N	+	NT	NT	+
2D5	N	+	NT	NT	+
18A3	N	+	NT	NT	+
12DI	N	+	NT	NT	+
9D1	N	+	NT	NT	+
14B5	N	+	NT	NT	+
14A4	N	+	NT	NT	+
4B4	N	+	NT	NT	+
14A6	N	+	NT	NT	+
12B3	N	+	NT	NT	+

+ = binding; − = no binding   NT = not tested

Table 1. Cross-neutralisation titres of Australian BEF virus strains and Berrimah virus, using polyclonal antibodies.

Ascitic fluid	BEF Virus			
	(BB7721)[1]	(CSIRO42)[2]	(CSIRO53)[2]	(DPP63)[1]
BEF BB7721	1024[3]	512	128	4
BEF CSIRO 42	64	384	8	<2
BEF CSIRO 53	512	1024	1024	32
Berrimah 1	192	32	32	4096
Berrimah 2	<2	6	4	384

[1] Bovine isolate
[2] Mosquito isolate
[3] Reciprocal of antibody dilution which neutralised 100 $TCID_{50}$ of virus in 50% of the wells
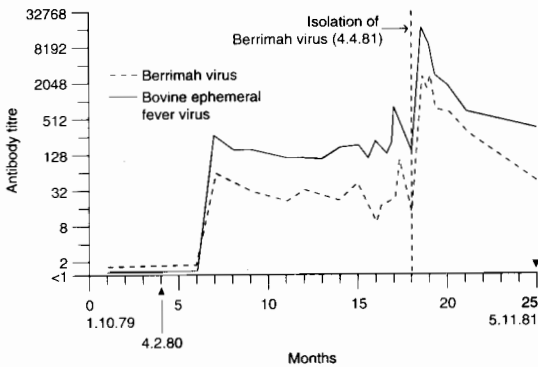
Fig. 1. Antibody titres to BEF and Berrimah viruses found in serial serum samples from a steer from which Berrimah virus was isolated.

It is thought that those antibodies which bind without neutralisation may act as blocking antibodies by binding to the virus and preventing or at least inhibiting the binding of neutralising antibodies. This may explain the mechanism by which Berrimah virus was able to escape neutralisation and cause an infection in the presence of high levels of neutralising antibody. Non-neutralising MAbs to the G, M2 and N proteins were tested by indirect immunofluorescence and the results are shown in Table 3. Of these, two MAbs did not bind to CSIRO 42 virus while five failed to bind to the CSIRO 53 isolate of BEF virus. Six non-neutralising G protein Mabs failed to bind to Berrimah virus.

The Beijing 1 strain of BEF virus was obtained in China, and processed by Peter Walker and Helen Zakrzewski. The virus was purified, the proteins

Strain variation using monoclonal antibodies

Monoclonal antibodies were prepared against the BB7721 strain of BEF virus and these reacted with the G, M2 and N proteins according to results obtained by polyacrylamide gel electrophoresis (PAGE) and immunoblotting (Tables 2 and 3). Fourteen of the MAbs against the BEF virus G protein were neutralising. Indirect immunofluorescence and neutralisation tests on ascitic fluid from these MAbs were used to determine their ability to bind and neutralise the two other Australian strains and Berrimah virus. The results are listed in Table 2. Only one MAb, 8D3, failed to neutralise CSIRO 42 virus while two MAbs, 8B6 and 3D6, failed to neutralise or bind CSIRO 53. Berrimah virus was neutralised by only eight of the neutralising MAbs. However, three additional MAbs were capable of binding to the virus without neutralisation.

separated by PAGE then transferred to nitrocellulose. In Australia, the virus protein preparations were tested by immunoblotting, for reactivity with 22 MAbs to the G, M2 and N proteins. Results are shown in Table 4. Four of the G protein-specific MAbs that bound to the BB7721 strain failed to bind to the Chinese strain.

Batch variation

To determine what effect, if any, passage level may have on antigenic variation, three batches of the BB7721 strain of BEF virus with different passage histories were tested against 14 neutralising MAbs. Batch 1 was passaged six times in mouse brain, 34 times in BHK 21 cells, twice in Vero cells then plaque cloned three times in Vero cells. Batch 2 received the same number of passages as batch 1 but was plaque

cloned separately. Batch 3 was passaged six times in mouse brain and 25 times in BHK 21 cells then cloned three times by limiting dilution in Vero cells. Batch 3 was not plaque cloned.

Neutralisation titres for the 14 MAbs against the three batches of BEF virus are listed in Table 5. Most MAbs gave the same titres with each batch of virus. However, batch 1 contained a proportion of virus which was partially resistant to the MAb 13C6, shown by virus breakthrough at low dilutions. Batches 2 and 3 were partially resistant to the MAb 5A5 while batch 2 was also partially resistant to the MAb 3D6.

Having demonstrated that various batches and isolates of BEF virus are not homogeneous, variants were isolated by incubating the BB7721 BEF virus with 13 of the neutralising MAbs and selecting plaques which escaped neutralisation. Escape frequencies for these variants, calculated as plaque forming units/ml in the presence of MAb divided by plaque forming units in the absence of MAb, are shown in Table 6. The average escape frequency was between 10^{-4} and 10^{-5}, although high escape frequencies were observed for 13C6, 3D6 and 5A5, while low escape frequencies were obtained for 16A6 and 8B6.

From the BB7721 parent strain of BEF virus, a total of 43 variants were selected which escaped neutralisation by the MAb used for selection and in most cases by other MAbs as well, resulting in 23 patterns of resistance as shown in Figure 2. To determine whether a variant was resistant or partially resistant, titres were compared with the titre of the parent virus. A virus was considered to be resistant to neutralisation only if the neutralising titre was less than two. In some cases, there was a greater than 10-fold reduction in the titre, which was considered to be partial resistance, although viruses showing only partial resistance to the selecting MAb were excluded from the study. It was assumed that when a mutant virus escaped neutralisation by a MAb, then the epitopes defined by these MAbs were functionally linked. In this way, patterns of neutralisation and resistance to neutralisation allowed the variants to be grouped into at least six clusters representing viruses with mutations affecting the same antigenic site.

Monoclonal antibody resistance in mice

MAb-resistant mutants were also detected in mice. Suckling mice inoculated intraperitoneally with neutralising or non-neutralising MAbs, then challenged intracerebrally with BEF virus, were generally protected from paralysis and death by the neutralising MAbs. Protected mice often survived longer than 14 days compared to 4 or 5 days for the controls. However, some mice survived no longer than the controls despite high levels of passive protecting antibody, indicating the presence of MAb-resistant virus (Table 7).

Fig. 2. Antigenic map of the BB7721 strain of bovine ephemeral fever virus. Neutralisation resistant variants were selected using monoclonal antibodies (MAbs), then tested for susceptibility (□) or resistance to neutralisation by the MAb used in selection of the variant (●) and by another MAb in the panel (★). (▲) denotes partial resistance. Labelling of antigenic sites as G1, G2, G3a, G3b is taken from Cybinski et al. (1990). Antigenic site G4 is a new site detected by competitive binding assay and by MAb resistance.

Table 4. A comparison of the Beijing strain 1 and the BB7721 strain of BEF virus by immunoblot analysis using monoclonal antibodies specific for the BB7721 strain.

Monoclonal antibody	Protein specificity	BB7721	Beijing 1
DB5	G	+	+
13C6	G	+	+
13A3	G	+	+
17B1	G	+	+
9C5	G	+	+
1C6	G	+	+
12A5	G	+	+
15B5	G	+	+
11D1	G	+	+
3D6	G	+	−
16A6	G	+	+
8D3	G	+	+
5A5	G	+	+
3A2	G	+	+
11B5	G	+	−
2C4	G	+	−
18C2	G	±	±
EB4	G	+	−
2C6	M2	+	+
FD2	M2	+	+
20A6	M2	+	+
11A3	N	+	+

+ = binding; − = no binding

Table 5. Neutralisation of BEF virus batches with monoclonal antibodies.

Monoclonal antibody	Neutralising titre[1]		
	BEF virus batch number		
	1	2	3
DB5	256	256	256
13A3	1024	1024	1024
13C6	16	4096	4096
17B1	>8192	>8192	>8192
9C5	256	256	256
1C6	256	256	256
15B5	256	256	256
12A5	1024	1024	1024
11D1	8	8	8
16A6	128	128	128
8D3	256	256	256
3D6	512	16	512
8B6	64	64	64
5A5	256	16	16

[1] Titres expressed as the reciprocal of the highest dilution required to neutralise 100 $TCID_{50}$ of virus in 50% of the wells

Discussion

Previous serological studies using polyclonal antibodies have failed to show any significant differences between strains of BEF virus from within Australia or in strains from different countries (Snowdon 1970; Inaba 1973; Tian et al. 1987). However, MAb studies on rabies and vesicular stomatitis virus have shown evidence of extensive antigenic diversity in these viruses (Wiktor and Koprowski 1980; Luo et al. 1988) and this diversity is thought to be one cause of vaccine failure for rabies virus.

The data presented here clearly indicate that considerable antigenic diversity also exists in BEF virus strains from different sources and that variants can be selected experimentally from cloned BEF virus either by passaging or by growing in the presence of MAb. The vaccine currently available in Australia is based on the BB7721 strain of BEF virus (Vanselow 1985) and this is known to be different from viruses now circulating (Cybinski et al. 1992). However, the viruses investigated shared the majority of epitopes as indicated by MAb reactions, therefore

Table 6. Frequency with which BEF virus (BB7721) escaped neutralisation by monoclonal antibodies.

Selecting monoclonal antibody	Escape frequency
DB5	10^{-4}
13A3	5×10^{-5}
13C6	6×10^{-3}
17B1	2×10^{-5}
9C5	4×10^{-5}
1C6	3×10^{-5}
15B5	10^{-4}
12A5	5×10^{-5}
16A6	5×10^{-7}
8D3	10^{-5}
3D6	$5 \times 10^{-3} / 10^{-5}$ *
8B6	5×10^{-8}
5A5	10^{-3}

* small plaques/large plaques

Table 7. Passive protection of mice inoculated with monoclonal antibodies and challenged with BEF virus.

Monoclonal antibody	Neut. titre	No.	Number of deaths recorded each day											
			4	5	6	7	8	9	10	11	12	13	14	>14
6.13C6	4096	7	—	—	1	1	—	2	—	—	—	—	—	3
6.17B1	2048	6	—	1	—	—	—	—	—	—	—	—	—	5
1.DB5	1024	7	—	—	—	—	—	—	—	—	—	—	—	7
4.13A3	1024	6	—	1	1	—	1	1	—	—	1	1	—	—
6.3D6	1024	5	2	—	—	1	—	1	—	—	—	—	1	—
4.1C6	256	9	—	1	—	—	1	1	—	2	—	—	3	1
4.12A5	256	6	—	—	1	—	1	—	—	—	1	—	—	3
4.15B5	192	6	—	—	2	1	—	1	1	1	—	—	—	—
6.9C5	128	7	—	—	2	4	1	—	—	—	—	—	—	—
6.8B6	16	6	—	1	1	—	2	2	—	—	—	—	—	—
5.8D3	8	7	—	—	4	1	1	1	—	—	—	—	—	—
1.16A6	8	7	—	1	4	2	—	—	—	—	—	—	—	—
6.11D1	4	6	—	5	—	1	—	—	—	—	—	—	—	—
6.18C2	<2	6	—	5	1	—	—	—	—	—	—	—	—	—
6.3A2	<2	6	—	6	—	—	—	—	—	—	—	—	—	—
6.18D2	<2	4	—	2	2	—	—	—	—	—	—	—	—	—
6.13B5	<2	6	1	2	3	—	—	—	—	—	—	—	—	—
5.11B5	<2	6	—	4	2	—	—	—	—	—	—	—	—	—
6.10B1	<2	7	4	2	1	—	—	—	—	—	—	—	—	—
5.2C4	<2	7	—	—	—	—	—	—	—	—	—	—	—	—
4.17D6	<2	6	2	3	1	—	—	—	—	—	—	—	—	—
Control	—	42	16	23	3									

— No deaths recorded

it is not surprising that the vaccine is protective. For the same reason, it is likely that vaccines against the BB7721 and the Beijing 1 strains of BEF virus would cross protect. On the other hand, there is at least one virus, Berrimah virus, which has a high degree of homology with BEF virus strains, but can escape neutralisation by high levels of BEF antibody. Berrimah virus is not known to be pathogenic but other pathogenic viruses could occur which avoid neutralisation by the same mechanism. These factors need to be considered when producing an ephemeral fever vaccine or a diagnostic test based on MAbs. Monitoring of current field strains of BEF virus is therefore essential.

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Appendix 1
Definitions

Ephemeral fever, BEF virus

In these Proceedings, the convention has been adopted to refer to the disease as bovine ephemeral fever (BEF) or ephemeral fever. The causative agent is described as BEF virus.

There are many citations of two isolates of bovine ephemeral fever virus in these Proceedings. Their provenance is as follows.

BB7721 (Australia)

A blood sample from a cow with clinical ephemeral fever at Charters Towers, Queensland, Australia, was inoculated into a susceptible steer at the Animal Research Institute, Yeerongpilly, Queensland. Blood taken from this animal was inoculated intracerebrally into suckling mice at the Queensland Institute of Medical Research, Brisbane (Doherty et al. 1968). The virus was subsequently adapted to growth in BHK21 cell cultures and has been used to produce both live and subunit vaccines.

Beijing or Beijing 1 strain (China)

The cow from which this virus was isolated had clinical signs of ephemeral fever in the 1976 epidemic in Beijing. Blood was passaged in suckling mouse brain until the death rate stabilised at the eighth passage level when the strain was designated as 76BAM. The virus was then adapted to BHK21 cell cultures and designated 76 BAMH. This strain has been used for various vaccines and for biochemistry and serological procedures. A fuller history is provided in these Proceedings by Liu Shangao of the Beijing Agricultural University.

Appendix 2

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Appendix 3
The 1st International Symposium on Bovine Ephemeral Fever and Related Rhabdoviruses

CHAIRMAN OF SESSONS

Day and Session Time	Co-Chairs
Thursday 25 August	
Opening Ceremony	Professor Chen Zhangshui
10.30–12.15	Professor Zhai Zhonghe, Dr D. Hoffmann
13.30–15.15	Professor Tang Guiyun, Dr C. Calisher
15.30–17.30	Professor Tang Guiyun, Dr C. Calisher
Wednesday 26 August	
09.00–10.15	Professor Bai Wenbin, Dr P. Daniels
10.30–12.15	Professor Bai Wenbin, Dr P Kirkland
13.30–15.15	Professor Zhou Weihan, Dr G Davies
15.30–17.30	Professor Zhou Weihan, Ms D Cybinski
Thursday 27 August	
09.00–10.15	Professor Guan Dapei, Professor J. Coetzer
10.30–12.15	Professor Guan Dapei, Dr T. St George
13.30–15.15	Professor Guo Yupu, Dr J. Cowley
15.30–Finish	Professor Guo Yupu, Dr D. Hoffmann

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