A Strain of Virus Isolated from *Culicoides homotomus*

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Abstract

A strain of RNA virus was isolated from *Culicoides homotomus*. The virus was spherical, unenveloped, 25–30 nm in diameter and produced at least six bands in RNA-electrophoresis gels. The virus produced cytopathic effects (CPE) in C6/36, Vero, BHK21, pharynx/larynx and chick-embryo fibroblast tissue cultures. It had an optimum growth temperature of 37°C, and was resistant to chloroform and ether, but sensitive to 0.25% and 0.5% trypsin. When injected into cattle and goats the virus produced homologous antibodies but no clinical signs. However, sheep developed fever when infected intravenously. The virus was re-isolated. After 12 passages, the virus could infect one- to two-day-old mice with classical signs and 80% mortality. The virus has not yet been identified.

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Aetiological and Epidemiological Studies on Ibaraki Disease in Taiwan

Lu Yongsiu, Li Yungkung and Liou Pepper*

Abstract

Nonsuppurative encephalitis has been reported sporadically in Taiwan since 1985. However, not until July 1990 was the causative agent recognised, when Ibaraki disease virus was first isolated from the cerebellum of a cow with non-suppurative encephalitis in the Chia-Yi district of Taiwan. During the period of the disease, an epidemiological survey was conducted on 163 young calves, aged less than one year, from 20 farms located in four towns in the Chia-Yi district. The results showed that 13 calves (7.9%) had suffered from the disease. Twelve sick calves were culled or died within a few days of the onset of clinical signs. Virus isolation was performed on blood samples from 73 calves. Overall, 28 strains of orbiviruses were isolated from plasma and 11 strains from blood cells. From September to October, six virus strains were isolated from the nasal discharges or blood of infected cattle, with symptoms mainly in the respiratory tract, from two farms in Pingtung. Viruses isolated from field cases were pathogenic to suckling mice, while experimental virus infections of cattle induced respiratory tract lesions. Virus was still recoverable from the blood of infected cattle 45 days after challenge. Over four consecutive years (1987–1990) a serological survey was carried out for antibodies to Ibaraki disease in cattle in Taiwan. The antibody-positive rate of dairy cattle in Taiwan increased from 25% in 1987 to 90% in 1990. These results indicate that the disease was epidemic in Taiwan's cattle population. This is the first report in Taiwan, and the third report (after Japan and Korea) in the world on clinical cases and virus isolation of Ibaraki disease in cattle.

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Epidemiological and Aetiological Studies on Chuzan Disease in Taiwan

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Abstract

Since 1985, many cases of nonsuppurative encephalitis and congenital abnormalities, of unknown aetiology, have occurred in cattle herds in Taiwan. In 1989, a serological survey was conducted in Taiwan on the prevalence of antibody to Chuzan disease. The antibody positive rate was 47%. A second survey in 1990 found the positive rate had increased to 84%. From December 1991 to October 1992, 26 calves with nervous signs and lameness were observed in dairy farms located in Tainan and Kaohsiung districts: 22 calves died within a few days of the onset of severe clinical signs. Virus isolation was attempted using heparinised blood samples collected from 12 sick calves. Three strains of virus were isolated. Using serological, biological, physical, and electron microscopical studies, these were identified as being Chuzan virus of the genus *Orbivirus*. Chuzan virus was first reported in Japan in 1985, where it caused an epidemic of congenital abnormalities with hydranencephaly/cerebellar hypoplasia syndrome of calves. This is the first report of Chuzan disease with the isolation of the Chuzan virus in Taiwan, and only the second report in the world.

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Sentinel Herds

Introduction

THE study of bluetongue epidemiology presents some unique difficulties. Overt disease affects only a small percentage of the vertebrate hosts of bluetongue virus. Many silent infections occur in cattle, buffalo, deer, antelope, goats and some breeds of sheep. These silent infections explain why bluetongue viruses, even when their presence is not perceived, are readily accessible for transmission by *Culicoides* vector species.

Another difficulty, referred to many times in these Proceedings, is that the disease is caused by at least 24 distinguishable viruses which form a related group with complex interrelationships. If all 24 bluetongue virus serotypes are available to a laboratory, it is simply a matter of routine to carry out 24 separate tests on serum samples collected in random surveys. Sera positive in the screening tests can then be titrated. But this all requires significant staff effort and expense. Sometimes this approach does give a clear answer, if just a single serotype is endemic in a particular area. More often, however, the presence of several cross-reacting serotypes means that a sophisticated mathematical analysis must be applied to assess the probabilities of individual serotypes being involved.

Silent infections with bluetongue viruses are readily detectable in endemic areas by taking regular and serial blood samples from healthy cattle, buffalo, goats or sheep, and then culturing for bluetongue viruses and testing for antibodies. Once the full range of bluetongue viruses that circulate in an area has been isolated from sentinel animals, retrospective and real time serological studies can unravel existing epidemiology and detect any appearance of additional serotypes on a real time basis. The outcome is a regularly updated guide as to which vaccines are necessary for protection of sheep in a given region. Virus-containing blood from healthy sentinel animals has been shown often to be fully virulent to sheep and so is valuable to validate experimental vaccines.

The sentinel herd technique that evolved in Australia has been successfully applied in Indonesia, Malaysia and in various provinces of China. Information on seasonality has yielded some unique observations in Inner Mongolia. With our emerging knowledge about the serotypes, it is time to coordinate research in the Asian-Pacific region, and to encourage countries that are not yet participating to define their bluetongue situation by establishing sentinel herds on a regional basis.

Australian National Arbovirus Monitoring Program—a Model for Studying Bluetongue Epidemiology in China

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Abstract

In Australia bluetongue viruses have not caused outbreaks of disease in sheep, but their presence continues to cause economic loss, especially in terms of reduced markets for the export of live sheep, cattle, semen and embryos. As some of the Australian serotypes of bluetongue virus (BLU) are capable of causing disease under experimental conditions, the sheep industry and animal health authorities are concerned that pathogenic BLU serotypes could enter major sheep-raising areas. This paper describes an extensive, nationwide sentinel herd program to monitor BLU epidemiology in Australia. This National Arbovirus Monitoring Program ensures the detection of any movements of bluetongue viruses towards the disease-free zone of southern and eastern Australia, and allows delimitation of virus-free zones from which animals can be safely exported. To assist with the development of disease control programs, a similar approach could be used to monitor the spread of bluetongue and other important vector-borne viruses in China and other countries.

AUSTRALIA'S international position as a supplier of live cattle, sheep and goats, and of ruminant germplasm, has been heavily dependent on the freedom of its national flocks and herds from major infectious diseases. Thus there was great concern about the threat to the livestock trade when, in 1977, a virus belonging to the bluetongue group was identified in Australia. Studies from 1978 to 1986 revealed the presence of another seven bluetongue virus (BLU) serotypes. The history of these virus isolations, and an overview of the epidemiology of bluetongue viruses in Australia, have been described previously (Gard et al. 1988; Gard and Melville 1989; Ward 1994). Detailed studies have also identified the biology and response to climatic variations of the major Australian BLU vectors.

Australia, like China, has large areas where there are sheep and few cattle. Australia's sheep are located mainly in the southern and interior parts of the country where there are no BLU vectors. Large desert areas separate the major sheep-raising zones from the northern vector populations. As well as the cold climate in the south and dry climate in the inland, these sheep-raising zones are also protected by oceans to the south, east and west. Consequently there are only relatively narrow pathways for the movement of vectors and their viruses into the sheep populations. Bluetongue virus infections have been largely confined to cattle and buffalo, mainly because there is no overlap in distribution between *Culicoides brevitarsis*, the major vector, and significant sheep populations. Climatic conditions restrict this vector to the northern parts of the country and to a narrow strip along the northern and central coastal regions of Queensland and New South Wales. In these latter areas, a few sheep have been infected but shown no evidence of disease. Nevertheless, the presence of these viruses in Australia continues to cause economic loss, especially in terms of reduced markets

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for the export of live sheep, cattle, semen and embryos.

As some of the Australian BLU serotypes are capable of causing disease under experimental conditions, the sheep industry and animal health authorities are concerned that pathogenic BLU serotypes could enter major sheep-raising areas. This threat is only likely under extremely favourable conditions for the vector, such as periods of high rainfall and mild autumn temperatures.

This paper reviews BLU epidemiology in Australia and describes a nationwide sentinel herd monitoring the spread of bluetongue and other important vector-borne viruses. A similar approach could be followed in China and other countries to monitor the spread of bluetongue and other important vector-borne viruses, thus helping the development of control programs and the identification of livestock populations free of arbovirus infections.

Materials and Methods

The objectives of this program were to:

- determine the annual distribution of BLU serotypes currently in Australia to provide an early warning of the movement of potentially pathogenic viruses southwards towards major sheep populations
- monitor any introduction of new BLU serotypes into northern Australia
- define regions free of bluetongue and Akabane virus infections to assist the export of livestock.

From 1984 to 1992, bluetongue infections in Australia were monitored by serology and virology studies in sentinel cattle. These studies were usually conducted and funded by State or Territory Departments of Agriculture or Primary Industries, with additional funds from farmers through the Meat or Wool Research Corporations. There were differences in the frequency and intensity of monitoring and, at times, significant gaps among the areas being covered.

In 1993, an integrated national program was established to monitor the spread of economically important insect-borne livestock viruses and their vectors. This new program is jointly funded by the livestock industries (the farmers) and the State and Federal (Australian) governments.

The National Arbovirus Monitoring Program (NAMP) is effected through the regular collection of blood samples from young cattle in sentinel herds and by the collection of insects with light traps. Most virus transmission shows a distinct seasonal pattern, occurring in summer and autumn and ended by the onset of winter in the temperate regions. Each year, therefore, the NAMP begins in the Southern Hemisphere spring (between August and October) and concludes at the end of autumn in the following calendar year.

In 1993–94, there were 62 insect collection sites and sentinel herds located throughout the distribution area of *Culicoides brevitarsis*, the main vector, and in the vector-free areas of the Australian mainland and Tasmania (Fig. 1). Light traps for insect collections are operated for three nights each month, and in most locations for eight months of the year, although some locations in the tropics are sampled all year round. Insects are collected into alcohol and the *Culicoides* species identified by entomologists in government laboratories in Western Australia, the Northern Territory, Queensland and New South Wales (NSW).

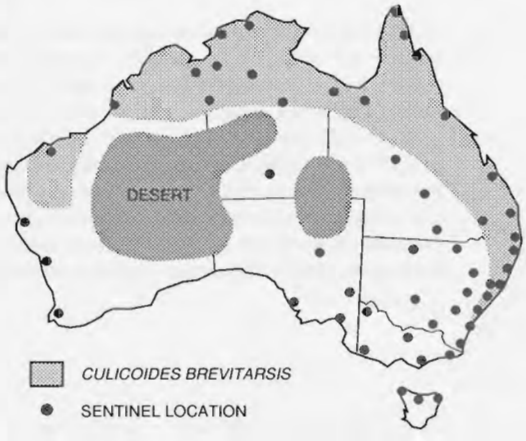

Figure 1. Location of sentinel herds and vector monitoring sites for National Arbovirus Monitoring Program (NAMP).

At the sentinel herd sites, a minimum of ten young cattle, usually five to seven months old, are bled once monthly for eight months of the year, beginning at the likely start of the vector season. Year round sampling, at monthly intervals, occurs at several tropical sites. At the Coastal Plains Research Station near Darwin in the Northern Territory, where most of the new BLU serotypes have been first isolated, a group of 20 sentinel animals are bled weekly for six months during the peak vector season. In vector-free areas, animals are sampled at the start and finish of each summer–autumn period.

Serum samples from sentinel animals are tested for BLU antibodies by competitive enzyme linked immunosorbent assay (cELISA) and, if positive, by virus neutralisation (VN) tests. Antibodies to Akabane and bovine ephemeral fever viruses are detected by VN.

Virus isolation is undertaken routinely on heparinised blood samples from the Coastal Plains sentinel herd near Darwin, Northern Territory, from

the time of the first bluetongue infections each season. For virus isolation, specimens are initially inoculated intravenously into 10-11-day-old chicken embryos. After seven days, the embryo homogenates are passaged onto mosquito cell cultures (*Aedes albopictus* C6/36) and then passaged up to three times onto BHK21 cells to detect any cytopathology due to virus replication.

Results

Between 1987 and 1993, most bluetongue surveillance in Western Australia comprised structured serological surveys in which single serum samples were collected. These surveys confirmed the presence of BLU serotypes 1 and 21 in known vector areas and freedom from infection in vector-free regions. In Queensland, similar surveys occurred until 1990, when sentinel herds were established. This monitoring showed that infection of cattle with BLU1 and 21 was relatively common in central and northern Queensland. Small numbers of sheep also appeared to be infected with BLU1 but there was no evidence of disease (Flanagan et al. 1993).

In New South Wales and the Northern Territory, sentinel animals were sampled in a similar distribution to that described above for the 1993/4 NAMP, but the number of sites and sampling frequency varied. In 1988, bluetongue infection was identified in sentinel cattle in the North Coast region of NSW. In 1989, infection was widespread along the coastal strip from north to south as far as Bodalla and inland through the Hunter Valley region on the Central coast. Significant numbers of sheep in very small flocks were infected in the Hunter and Manning River Regions but showed no evidence of disease. In both years, the transmitted virus was BLU1.

In the Northern Territory, bluetongue virus infection has been found in sentinel cattle each year except 1990. The level of activity and number of serotypes transmitted has varied from year to year. In 1987, only BLU3 was identified, with BLU1 and 16 in 1988, and BLU1, 3 and 23 in 1989. In 1991, only BLU3 was identified, while in 1992 animals were infected with BLU16 and 20. This latter was the first occasion on which BLU20 had been isolated and identified since the original isolation of bluetongue virus in 1975 (St. George et al. 1978), suggesting that this serotype may have died out in Australia in the interim and subsequently re-entered the country.

During the 1993/4 arbovirus year, the transmission patterns of the three arboviruses of interest to the NAMP were identified as follows.

Bluetongue viruses

There was evidence of BLU transmission in four states; Western Australia, Northern Territory, Queensland and NSW. Although transmission occurred over a large geographical area, infection was still confined to known vector areas (Fig. 2).

In the Northern Territory, BLU seroconversions were only recorded in the sentinel herds at Berrimah and Coastal Plains, both near Darwin. Transmission occurred between January and March 1994. Only BLU1 appeared active: there was no detectable transmission of other serotypes, including no evidence of the introduction of new serotypes.

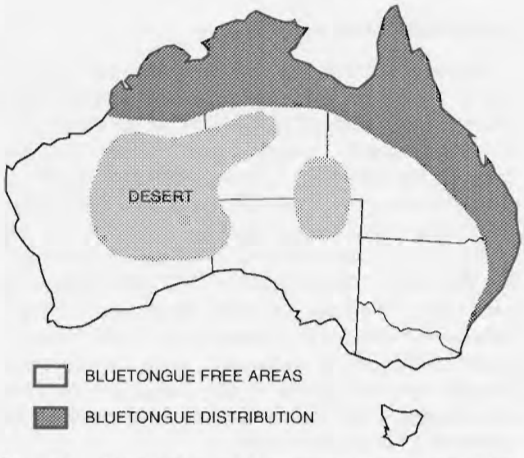

Figure 2. Distribution of bluetongue viruses and bluetongue-free zones in Australia, 1994.

In the north-west of Western Australia, at Kununurra, seroconversions occurred each month between November 1993 and January 1994 inclusive. At Kalumburu, seroconversions occurred in August 1993 and April 1994. Only BLU1 was being spread in Western Australia. The southern vector-free regions of Western Australia were free of bluetongue virus infection.

In Queensland, seroconversions were recorded in October 1993 at Etna Creek (near Rockhampton) and between November 1993 and April 1994 in herds in the far north and on Cape York. In southern Queensland, seroconversions occurred at Dalby and Maryborough in April. There was evidence of infection with BLU21 in the herds on Cape York and Northern Queensland at Utchee Creek, although there was also infection with BLU1 at Utchee Creek and among the herds in Southern Queensland. These are all areas where there are very few sheep.

In NSW, bluetongue transmission appeared to spread from a focus on the mid-north coast at Coffs Harbour in December 1993, moving mainly southwards, and eventually reaching Camden, where seroconversions were recorded in early June, consistent with infection during May. Infection was first recorded on the NSW far-north coast in June, with a

low level of infection at Lismore. Herds on the mid-north Coast and in the coastal Hunter–Manning regions showed high rates (60–100%) of seroconversion. Infection, shown to be caused by BLU1, spread west as far as Scone on the Upper Hunter Valley. At the end of the season, bluetongue antibody was present in some 63% (14/22) of sentinel sheep at Gloucester, although there were no reports of disease. The prevalence of Akabane antibody in these sheep was more than 95% (21/22), confirming a high attack rate from *Culicoides* spp.

Bovine ephemeral fever virus

Bovine ephemeral fever virus (BEF) has a largely similar pattern of distribution to bluetongue virus. There is a reservoir of infection in the northern areas and the mosquito vectors move the virus into the south in the summer. Although the main vector is probably a mosquito, this virus is usually confined to areas where bluetongue and Akabane viruses are found, that is, areas where *C. brevitarsis* is common. In 1994, there was evidence of BEF transmission in four states; Western Australia, Northern Territory, Queensland and NSW. However, in NSW, where a mosquito vector is suspected, infection occurred beyond the usual limits of the *Culicoides* endemic area. Significant disease occurrence was noted in the Northern Territory and NSW.

In the Northern Territory, BEF infection was detected in all northern herds with infection likely between January and March 1994. The sentinel herd at Alice Springs, near the centre of Australia, remained free of infection. In Western Australia, in the far north-west at Kalumburu, BEF seroconversions were recorded in August and October 1993.

In Queensland, sentinels were infected in the Gulf of Carpentaria and Cape York at various times between December 1993 and May 1994. At Utchee Creek BEF transmission occurred in January. In south-east Queensland transmission occurred at a low level between January and April 1993.

In NSW there was extensive BEF transmission, commencing in the Hunter–Manning region on the coast in the middle of the state and on the far-north coast. The disease was particularly severe at Paterson in the Hunter Valley, with 9/10 sentinels very sick and all seroconverting within one month. The other notable feature was the spread up the Hunter Valley beyond Scone and to Dubbo, about 400 km inland.

Akabane virus

There was generally a lower level of Akabane infection than usual, and less than the other viruses being investigated. In Western Australia the herd at Kalumburu showed evidence of Akabane infection in August and October 1993, while in the Northern Territory seroconversions occurred in three herds between January and March 1994. In Queensland sporadic infections occurred in most locations between December 1993 to February 1994 and April to May 1994. Infections were detected between February and June 1994 in the northern coastal herds in NSW, with transmission occurring as far south as Camden. All Akabane virus transmission occurred within the arbovirus endemic area, that is, within the range of the principal vector *C. brevitarsis*. The inland and southern areas of NSW and all southern states remained free of infection.

Discussion

In Australia, there is continuing economic loss because of the presence of several arboviruses which infect livestock. Akabane and bluetongue viruses, because of their potential to cause disease outbreaks and because of the continuing disruptions to trade, are of concern to the cattle, sheep and goat industries.

In the case of sheep, a significant feature affecting the complex interaction between these viruses and their mammalian hosts is the significant separation between the national sheep flock and relevant vector populations. Although disease caused by Akabane virus has occurred in sheep in NSW, only relatively small numbers of sheep live within the vector zone with virtually none at the critical stage of gestation (during which viral infection causes teratogenic effects) during the vector season. However, each year, Australian access to valuable export markets for the sale of live cattle and sheep, and embryos and semen, is denied because of the presence of Akabane virus in the country.

Although there have been cases of sheep being naturally infected with bluetongue virus in small flocks in New South Wales and Queensland, these have not been near the principal sheep areas, nor has there been any disease. The NAMP results for 1993/94 confirm that BLU serotypes with pathogenic potential appear still to be confined to a discrete area in the Northern Territory, extremely remote from the commercial sheep areas, and there is no evidence of the entry of new BLU serotypes. Unless a more pathogenic strain of virus were to be transmitted in the temperate zones of either eastern or western Australia, the risk of an outbreak of bluetongue disease appears to be low. Nevertheless, as with the Akabane situation, disruptions to trade continue.

However, the NAMP is able clearly to define the limits of both these important arboviruses and their vectors, and to identify distinct seasonal patterns of transmission. As a consequence of establishing both patterns and times of transmission, it is possible to identify the sheep populations most at risk of disease if a pathogenic virus were to spread southwards

(although the latter has not happened so far). As a corollary to the above, it is possible to certify, with reliability, geographical areas that are continually free from bluetongue and Akabane viruses and vector activity, and to define the times at which there is no risk of infection in areas where there is seasonal arbovirus transmission. These patterns of virus and vector spread, especially along the key southern interface with major sheep and cattle populations, have been developed from information gathered in intensive studies over more than 20 years. The vector- and virus-free areas can therefore be described very accurately, and export shipments of either livestock or germplasm can be made from these areas with a high degree of confidence. Large populations of livestock (more than 30 million sheep) previously described as being located within a vector area have now been shown conclusively to be free of arbovirus infections: these populations should now be eligible for inclusion in export shipments.

When individual sentinel herds are sampled, useful information can be obtained about the epidemiology of vector-borne viruses, such as bluetongue and Akabane, on a local basis. However, samples obtained from a regional, or preferably nationwide, network of coordinated sentinel herds provide much more valuable data. Such a system is recommended for a country where the epidemiology of an arbovirus disease has not been studied in detail. In China, for example, understanding the movements of viruses such as bluetongue may provide a guide to the times and places when a disease outbreak will occur, and may allow identification of serotypes of greatest threat. In turn, this may allow initiation of a strategic, preventative vaccination program to control disease in the most economical and efficient manner. In areas where there may be a restricted number of serotypes, the need for vaccines against only one or two serotypes can be predicted. Efforts to establish regional and national networks of sentinel herds should therefore be encouraged actively.

Acknowledgments

Many farmers throughout Australia have given outstanding co-operation for nearly 20 years, which has been essential for the success of the monitoring studies. Funding has been provided by all state governments, the Australian federal government, Australian Meat Research Corporation and Australian Wool Corporation.

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Recent Experiences with the Monitoring of Sentinel Herds in Northern Australia

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Abstract

From 1990 to 1995, sentinel herds have been monitored regularly at six locations throughout the Northern Territory of Australia. At all sites, monthly serology has been conducted for the bluetongue (BLU), epizootic hemorrhagic disease (EHD) and Palyam group viruses. Weekly virus isolation was also carried out at the site of greatest known arboviral activity. During the observation period, five BLU serotypes, five EHD serotypes, three Simbu serogroup viruses, one bovine ephemeral fever (BEF) serogroup virus and other arboviruses were isolated from the sentinel cattle. There was marked annual variation in the total number and identity of viruses isolated. Seroconversions in the sentinel herds also showed annual variation.

THE isolated bluetongue viruses have been studied molecularly: cDNA was prepared from isolates of each serotype of each year, and a fragment from the genome segment RNA3 was amplified by PCR. Nucleotides of these fragments have been sequenced and the sequences compared with the prototype Australian strain for each serotype. While for most isolates there was little variation from known sequences, in the case of recent isolates of BLU20 and 21 there were differences greater than 5%, indicating strains of viruses not encountered previously.

A bluetongue virus (BLU), serotype 20, was first isolated from *Culicoides* collected in the Northern Territory of Australia in 1975 (St. George et al. 1978). In the eleven years to 1986, a further seven serotypes were isolated (Gard et al. 1988). Since then no other serotypes have been isolated, although various combinations of the previously identified serotypes have been isolated in most years (Gard and Melville 1992). Currently eight serotypes have been identified, BLU1, 3, 9, 15, 16, 20, 21 and 23.

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The first BLU isolation came from insects, *Culicoides* species., which were being collected in conjunction with serological monitoring of a sentinel herd of cattle. The discovery of a bluetongue virus resulted in intensification of the sentinel program, and the BLU serotypes subsequently identified were isolated from sentinel cattle (St. George et al. 1980; Gard et al. 1988). Since 1990, the sentinel herd program for arbovirology has been part of the Northern Australian Quarantine Strategy (NAQS), and in 1994 the NAQS arbovirology program was incorporated into the National Arbovirus Monitoring Program (NAMP). This paper reports the results of sentinel herd monitoring in the Northern Territory from 1990 to 1995, primarily conducted as a component of NAQS, but supplemented by other programs for the characterisation of isolates.

The Sentinel Program

The sentinel program has had three primary objectives:

- isolation of arboviruses, particularly bluetongue viruses, that are active each year;
- serological monitoring of the annual distribution of major arboviruses; and
- serological monitoring of seasonal patterns of infections.

Virus isolation of arboviruses

The Coastal Plains Research Station (Fig. 1), site of the initial isolation of a BLU virus and most subsequent isolations, has been chosen as the sampling site representative of northern Australia. Past experience has shown that a greater range of viruses can be isolated here than at the other Northern Territory sites sampled to date. Monitoring occurs to detect arboviruses new to Australia, and to identify which of the known viruses are circulating each year at the sentinel site.

Serological monitoring of annual distribution of major arboviruses

There are also sites at locations representative of large pastoral areas or of other places important to the livestock industries (Fig. 1). Thus a site at the Berrimah Agricultural Research Centre is important: as other BLU research is conducted there it is essential to monitor its background arbovirus activity. In addition, Berrimah Agricultural Research Centre is adjacent to the port of Darwin, through which livestock are exported. Sites at the Douglas Daly Research Farm, Katherine Research Station and Victoria River Research Station monitor for the distribution of viruses to the south, south-east and south-west respectively. At various times, sites further to the south-east have been monitored for the spread of bluetongue viruses beyond their normal range (Newcastle Waters, Macarthur River and Rockhampton Downs, which is the current site). The Arid Zone Research Institute in the far south of the Northern Territory monitors an area usually free of arboviruses.

Serological monitoring of seasonal patterns of infections

All sites have been sampled monthly for serology, to determine not only the distribution of infections each year, but also the seasonal pattern of those infections. Knowledge of seasonal patterns is important epidemiologically to aid an understanding of the conditions under which the risk of spread increases. Similarities and differences among the sentinel sites, and among the patterns of distribution of the viral groups, can also be identified.

Sampling protocols

Samples of heparinised blood for virus isolation have been collected weekly at Coastal Plains Research Station. Sera samples for serology have been collected monthly from most sites, although some sites in the far south and south-east may be sampled only every three months, to reduce costs.

Test procedures

Samples for virus isolation were processed through embryonated chicken eggs, mosquito cell (C6/36) cultures and mammalian cell cultures (Gard et al. 1988). Sera were tested by agar gel immunodiffusion (AGID) for antibodies to the bluetongue, Simbu (Akabane, AKA), bovine ephemeral fever (BEF), epizootic hemorrhagic disease (EHD) and Palyam groups of viruses. In recent years, use of the more specific competitive enzyme linked immunosorbent assay (cELISA) for BLU group antibodies (Lunt et al. 1988) has replaced AGID testing for that serogroup. Microtitre serum neutralisation tests have been used when more serotype-specific information has been required for any of the viruses.

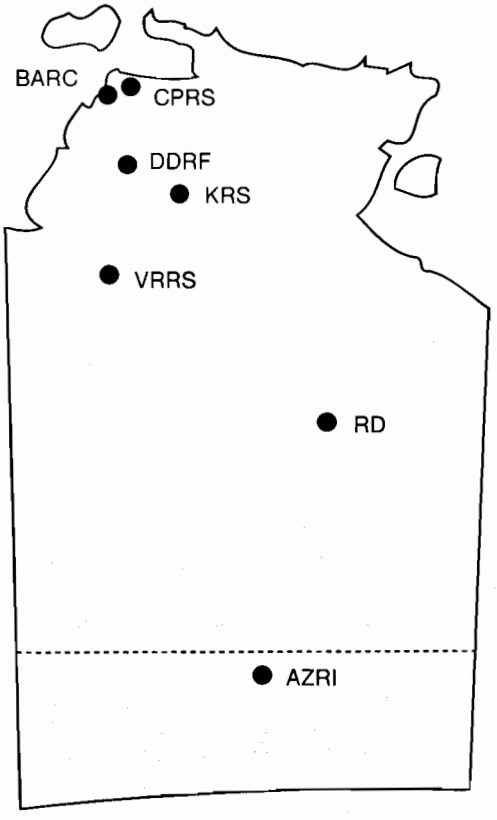

Figure 1. Sites of sentinel cattle herds monitored for arbovirology in the Northern Territory: BARC, Berrimah Agricultural Research Centre; CPRS, Coastal Plains Research Centre; KRS, Katherine Research Station; VRRS, Victoria River Research Station; RD, Rockhampton Downs; AZRI, Arid Zone Research Institute.

Molecular epidemiology

Recently, comparisons among isolates based on genotype have introduced an extra dimension to sentinel studies. A specific fragment of BLU viral RNA segment 3 has been amplified in polymerase chain

reactions (PCR) (Saiki et al. 1988), using primers described by McColl and Gould (1994). The nucleotide sequence of the amplified products has been determined by dideoxynucleotide chain termination sequencing (Sanger et al. 1977).

Nucleotide sequence alignments were carried out using the ALIGN Plus Program Version 2.0 (Scientific & Educational Software). SEQPROG (Knowles, unpublished) was used to compare the sequences using DNADIST and KITSCH programs from the PHYLIP package (Felsenstein 1985) and to give a single most parsimonious, unrooted tree. Basically, isolates were assigned to groups on the basis of aligned sequences showing 95% or greater homology.

Isolation of viruses at Coastal Plains Research Station, 1990–1995

During this six year reporting period, five BLU serotypes were isolated at Coastal Plains Research Station (Table 1). The year 1990 was unusual, with no BLU being isolated. Five EHD serotypes (1, 2, 5, 7 and 8) were isolated, as well as the Simbu group viruses Akabane, Aino and Peaton. The isolation of EHD1 was the first time this virus had been detected in Australia. Another new virus, of the Bunyamwera serogroup of the Bunyaviridae, was also isolated, as were other viruses which remain to be fully characterised.

Table 1 also shows the month in which each BLU isolation occurred at Coastal Plains Research Station. In 1991 isolations began late in the season, in May, while in 1992, 1994 and 1995 BLU isolations occurred throughout the wet season, from January to May.

Although BLU1 occurred in two consecutive years, 1993 and 1994, as did BLU21 in 1994 and 1995, the serotypes isolated each year could not be predicted from the previous year's data. BLU3 and BLU16 appeared for one year only. BLU20 appeared in 1992, then again in 1995. The isolations of BLU20 in 1992, the first since the original isolation in 1975 (St. George et al. 1978), came from only two of a total of 75 animals being monitored for various experimental purposes. Subsequent serum neutralisation testing showed those two to have been the only animals infected with that serotype, whereas 59 of the 75 yielded isolates of BLU16 that same year.

The data highlight our present poor understanding of BLU epidemiology at even such a well monitored site as Coastal Plains Research Station. There is no predictive data and no proven explanations for the serotypes that are active each year, the months in which they infect monitored animals, or the proportion of animals that become infected. New strategies are required to study BLU epidemiology more completely.

Similar patterns were evident among the EHD viruses which were isolated (data not presented). In 1990, when no bluetongue viruses were isolated, three EHD serotypes were recovered: EHD2 was isolated from 44 of 58 animals being monitored, EHD5 from 20 animals and EHD8 from five animals. Such data suggest that vectors were sufficiently abundant to maintain cycles of infection.

Seroepidemiology

At some sentinel sites, seroconversions to the various viral groups were recorded each year (Table 2). Palyam group viruses tended to be more widely distributed than BLU and EHD viruses, as indicated by the data from Katherine Research Station in 1991 and 1992 and Victoria River Research Services in 1991, 1994 and 1995 (Table 2). From the data, Katherine Research Station and Victoria River Research Services approximately mark the normal boundary of BLU activity, depending on the year. Arid Zone Research Institute showed no reactions to orbiviruses throughout the observation period, confirming its status as usually virus-free.

Molecular epidemiology

The BLU isolates were studied by sequence analysis of a PCR-amplified fragment of the RNA 3 gene coding for the inner capsid protein VP 3 (Table 3). The isolates included one of each serotype from each

Table 1. Isolations of bluetongue viruses at Coastal Plains Research Station, 1990 to 1995, by month.

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
1990	–	–	–	–	–	nt	nt	nt	nt	nt	nt	nt
1991	–	–	–	–	3	3	–	–	–	nt	nt	nt
1992	16	16	16	16	16,20	–	–	–	–	–	nt	nt
1993	–	–	1	1	1	–	–	–	–	nt	nt	nt
1994	1	1	1	1	21	21	–	–	–	21	21	21
1995	21	21	21, 20	20	20							

nt: not tested

year, with the exceptions of BLU1 in 1993 and the 1995 isolates of BLU21. Both isolates of BLU20 in 1992 were studied, as were both isolates of BLU21 from 1994. Computer-assisted groupings are presented in a dendrogram (Fig. 2).

Topotyping of Australian BLU isolates, based on analysis of sequence data, began when Gould (1987) reported that the prototype strains of BLU1 and 9 grouped together, showing less than 5% divergence of sequence homology, while BLU15 was 20% different from the other isolates.

The dendrogram (Fig. 2) shows the relationships of recent isolates (Table 3) with Australian prototype isolates for each serotype. With the exception of BLU15 (B15 Aus, Fig. 2), all prototype serotypes grouped together, as reported previously (Pritchard et al. 1995). Grouping with these prototypes were the isolates V2115, a 1991 isolate of BLU3; V2208, a 1992 isolate of BLU16; V3036, a 1994 isolate of BLU1; and V3209, a 1994 isolate of BLU21. These isolates of BLU1, 3, 16 and 21 can be assumed to represent strains of viruses already adapted to Australian conditions and evolving with the prototype strains.

As reported by McColl et al. (1994), the BLU20 isolates from 1992 are clearly different. These isolates may represent a strain of BLU20 that has evolved separately from the known Australian strains (Gorman et al. 1981), the inference being that they are recent incursions to northern Australia. The 1995 isolate of BLU20, V3594, was different by a similar order of magnitude from both the prototype and the 1992 isolates of BLU20, again indicating an incursion from a different source. The 1995 isolate grouped with an isolate of BLU1 from Malaysia, indicating homology with viruses from a source in Southeast Asia.

Another interesting observation was the comparison of the sequences of two isolates of BLU21 from 1994. Whereas V3209 grouped with the Australian prototype isolates, V3217 showed a sequence markedly different from that of all other isolates made in Australia, and more closely related to an isolate of BLU1 from India. Both the 1994 isolates of BLU21 studied were recovered from sentinel cattle at Coastal Plains Research Station within a month of each other. Hence the data indicate that two separate strains of BLU21 were circulating among the sentinel cattle at Coastal Plains Research Station at that time.

Table 3. Bluetongue isolates sequenced for molecular epidemiological study.

Isolate	BLU serotype	Collection date
V2115	3	May 1991
V2208	16	February 1992
V2400	20	May 1992
V2450	20	June 1992
V3036	1	February 1994
V3209	21	May 1994
V3217	21	June 1994
V3594	20	March 1995

There is much to be learned about the biological causes for the differences detectable among the nucleotide sequences of BLU isolates, and particularly the rate of change in sequence for any strain. Full epidemiological interpretations of the data must wait upon such basic knowledge. However, the detectable differences in sequence among isolates do provide a valuable measure of difference that is immediately useful. Detection of a sequence different from that usually encountered at any location signals a change in the viral fauna, most probably the result of an introduction of new genes in new viruses. New genetic material may equate with new, or increased, risks. For example, the new viruses may have different relationships with the vector species present, and hence may spread more slowly or more rapidly than pre-existing strains. The new viruses may be more pathogenic. The detection of a different genotype warns of a new situation to be monitored, and is also one of the means of undertaking that monitoring.

Table 2. Sentinel sites showing seroconversions to orbiviruses in agar gel immunodiffusion (AGID) serogroup tests[a].

Sentinel Site	1991 B E P[b]	1992 B E P	1993 B E P	1994 B E P	1995 B E P
Arid Zone Research Institute	− − −	− − −	− − −	− − −	− − −
Berrimah Agricultural Research Centre	− + +	+ + +	+ + +	+ + +	+ + +
Coastal Plains Research Station	+ + +	+ + +	+ + +	+ + +	+ + +
Douglas Daly Research Farm	− + +	+ + +	+ + +	+ + +	+ + +
Katherine Research Station	− − +	− − +	+ + +	+ + +	+ + +
Victoria River Research Services	− − +	− − −	+ + +	− − +	− − +

[a] cELISA was used for BLU serogroup testing in 1993, 1994 and 1995.
[b] B – BLU; E – EHD; P – Palyam.

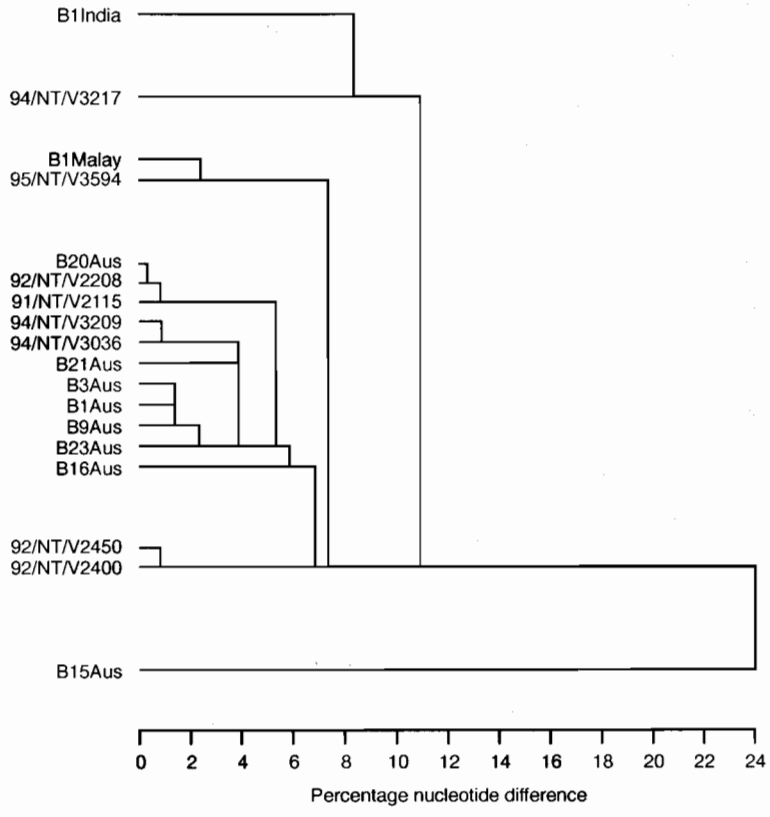

Figure 2. Dendrogram showing the relationships among recent Australian bluetongue isolates, as determined by nucleotide sequence analysis.

Conclusions

Although recent monitoring of sentinel herds in the Northern Territory has not yielded isolations of BLU serotypes additional to those known since 1986 (Gard et al. 1988), valuable information has been obtained. The unpredictability of arboviral infections has been emphasised, highlighting the need for new epidemiological studies to increase our understanding of BLU epidemiology. New viruses such as EHD1 and the Bunyamwera serogroup virus have been isolated, reinforcing our awareness that northern Australia is part of a broader East Asian ecosystem for arboviruses, and thus subject to introductions of new viruses on a continuing basis. That this is true for bluetongue viruses was identified by genotype analyses of recent BLU isolates, where serotype has suggested an apparently familiar phenotype. Nucleotide sequence analysis has identified several isolates of BLU20 and 21 that are new to Australia. In future, effective monitoring strategies must attempt to apply newer approaches, both in Australia and in the region, to detect changing risks, and to develop the capacity to analyse changes so as to develop appropriate responses.

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Establishment of Sentinel Herds to Monitor Bluetongue in China

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Abstract

Sentinel herds have been used in Australia since 1969 to monitor endemic virus infections of livestock. The technique, developed in Australia, has since been introduced to New Zealand, Papua New Guinea, Indonesia, Malaysia, USA, Canada, Central America and the Caribbean. In 1984, sentinel herds were established for a Sino-Australian ephemeral fever project in northeast China. In 1991, the first sentinel group for bluetongue, a disease first recognised in China's Yunnan Province in 1979, was established near Kunming. The herd comprised 15 goats and two cattle. This paper reports the establishment and operation of three more sentinel herds in Yunnan, in Shizong County and at two sites in Eshan County (12 cattle, 10 cattle and 15 goats respectively).

BLUETONGUE is an arthropod-borne viral infection, transmitted by biting insects. As bluetongue virus (BLU) does not cause obvious clinical disease in cattle, buffalo or goats, serological methods must be used to determine if animals have been infected. Viraemic animals introduced to an area where *Culicoides* vectors are active may be a source of infection for susceptible sheep. One way of monitoring BLU activity in an area is to establish sentinel animals, then test their sera for antibodies and isolate viruses from their blood. An understanding of virus distribution and activity can help in planning control measures.

Bluetongue disease was first discovered in China in 1979, in Yunnan Province, with serotype BLU1 being isolated from sick sheep (Zhang Nianzu et al. 1989a). During the subsequent nationwide bluetongue survey, antibodies were found in most provinces of China. BLU1 was later isolated from sentinel goats at Xinjiang and Inner Mongolia and from sick sheep at Shanxi, while BLU16 was isolated from sheep at Sichuan and Hubei.

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Materials and Methods

To investigate the distribution of BLU serotypes, four sentinel herds were established in Yunnan Province; at Kunming, Eshan (two sites) and Shizong. These herds, comprising two groups of 15 goats, one group of 10 cattle and another of 12 cattle, were regularly bled for serology and virus isolation.

Preliminary survey to select sites for sentinel herds

Before the establishment of sentinel herds, blood samples were taken from cattle, buffalo, goats and sheep in the candidate villages and tested for BLU antibodies. This involved the testing of 47 animals (30 goats and 17 cattle) from Shuanglong Village in Kunming, 94 (40 goats, 15 sheep, 17 cattle and 22 buffalo) from Shizong County and 104 (54 goats, 22 cattle and 28 buffalo) from Eshan County. Sera were

tested by competitive enzyme linked immunosorbent assay (cELISA) and agar gel immunodiffusion (AGID): positive sera were tested with standard neutralisation tests using Chinese isolates of BLU1 or BLU16.

Sentinel animals

Overall, four sentinel herds were established (Table 1). In September 1994, a sentinel herd was established at Shuanglong Village, 30 km north of Kunming (altitude 2000 m above sea level). The herd comprised 16 goats and 2 cattle aged under one year, all tested negative for bluetongue by AGID and cELISA. Blood samples were taken weekly from September to October 1994 and monthly from November 1994 to July 1995.

Table 1. Location, composition and date of establishment of sentinel herds

Location	Date of establishment	No. of cattle	No. of goats
Kunming	9 September 1995	2	15
Shizong	13 July 1995	12	0
Eshan 1	28 February 1995	10	0
Eshan 2	1 May 1995	0	15

In 1995, another herd, comprising 10 local Yellow cattle, was established at Baoqian Village in Eshan County, 150 km south of Kunming (altitude 1400 m; average annual rainfall 1041 mm; average annual temperature 16.2°C). Blood samples were taken monthly from 28 February to April 1995. In May 1995, 15 goats were substituted for the positive sentinel cattle and weekly blood samples were taken from May to July 1995.

To establish another sentinel herd, 12 local Yellow cattle bought from the sentinel village at Shuanglong (see above) were relocated to Wulong Village in Shizong County, 260 km east of Kunming (altitude 1600 m; average annual rainfall 1800 mm; average temperature 13.5° C). Weekly blood sampling began on 19 July 1995.

Animals were bled using evacuated bleeding tubes with 1.2 × 40 mm needles. Sera and clots were separated, numbered and recorded. Sera were stored in a serum bank, and blood and clots held at 4°C for virus isolation.

Serology

Sera from the sentinel herds were tested using AGID (Zhang Nianzu et al. 1989b), with antigen made from BLU1 (YF8). Reference sera were made at the Yunnan Tropical and Subtropical Animal Virus Diseases Laboratory. The sera were also tested by cELISA, as described by Gard and Kirkland (1993), using kits produced by Tropical Biotechnology, Townsville, Queensland, Australia. Microneutralisation tests were carried out by modified standard procedures (Gard and Kirkland 1993).

Virology

Heparinised blood and clots from preliminary survey samples, and from monthly or weekly samples of sentinel herds in Kunming, Shizong and Eshan, were processed for virus isolation. Heparinised blood (0.1 mL) or uncoagulated red cells (0.1 mL) from a clot were diluted in 0.9 mL sterile distilled water (pH 7.2–7.4) to lyse blood cells. Four 10-day-old embryonated hen eggs were inoculated intravenously (0.1 mL of inoculum per egg). Eggs dying one to five days post-inoculation were harvested separately from embryos alive after five days. The heads and legs of the embryos were removed, the body ground in a tissue stomacher with 5 mL diluent, and the supernatant stored in centrifuge tubes for inoculating cells.

Results

Preliminary survey

The preliminary survey involved the testing of blood samples from 245 animals in Kunming, Shizong and Eshan. While all 47 animals from Kunming were negative for BLU by AGID and cELISA, some animals from both Shizong and Eshan tested positive (Tables 2 and 3 respectively).

Table 2. Bluetongue serological survey results from the Shizong sentinel site.

Species	Total no.of animals	cELISA positives	AGID positives	Serum neutralisation positive serotypes		
				BLU1	BLU15	BLU16
Buffalo	22	18	13	3	0	0
Cattle	17	10	5	1	0	0
Goats	40	9	1	0	0	0
Sheep	15	0	3	0	0	0
Total	94	37	22	4	0	0

Table 3. Bluetongue serological survey results from the Eshan sentinel sites.

Species	Total no. of animals	cELISA positives	AGID positives	Serum neutralisation positive serotypes		
				BLU1	BLU15	BLU16
Buffalo	28	21	19	14	3	4
Cattle	22	18	6	0	0	0
Goats	54	6	4	0	0	0
Total	104	44	29	14	3	4

Sentinel herds

Of the 10 cattle at Eshan, five were positive for BLU antibodies by AGID (four weak positive and one strong positive) and seven positive by cELISA (Tables 4a and 4b). Three of the ten were seronegative by AGID and cELISA. The positive animals were all older than one year while the negative ones were all aged from 0.6 to one year. Because most of the sentinel cattle had BLU antibodies, an additional group of 15 BLU-negative sentinel goats were located at the same village in May 1995 and bled weekly. Until 2 June, all goats remained negative by AGID and cELISA. Virus isolations from blood and clot samples from the Eshan herds have not yet been completed. Lysed blood has been inoculated into embryonated hen eggs.

Table 4a. Serological results from the Eshan sentinel cattle, 28 February to 19 May 1995.

Date	28 February 1995		28 March 1995		28 April 1995		12 May 1995		19 May 1995	
Animal no.	cELISA	AGID	cELISA	AGID	cELISA	AGID	cELISA	AGI	cELISA	AGID
1	95	1	199	1	199	9	NT	0	NT	3
2	0	0	0	0	0	0	NT	0	NT	0
3	0	0	0	0	0	0	NT	0	NT	0
4	100	1	100	2	100	2	NT	0	NT	2
5	0	1	0	0	0	0	NT	1	NT	0
6	93	1	100	1	100	1	NT	0	NT	3
7	100	1	100	1	100	1	NT	0	NT	2
8	92	1	100	1	100	1	NT	1	NT	1
9	96	1	100	0	100	0	NT	1	NT	1
10	98	0	100	0	100	0	NT	0	NT	1

NT=not tested

Table 4b. Serological results from Eshan sentinel cattle, 26 May to 23 June 1995 (cELISA tests not completed).

Date	26 May 1995	2 June 1995	9 June 1995	16 June 1995	23 June 1995
Animal no.	AGID	AGID	AGID	AGID	AGID
1	0	0	1	1	1
2	0	0	0	0	0
3	0	0	0	1	0
4	0	1	1	1	2
5	0	0	0	0	0
6	2	2	2	3	3
7	0	0	1	0	2
8	0	1	1	1	1
9	0	0	0	1	1
10	0	2	1	0	1

The sentinel goats and cattle at Shuanglong Village in Kunming were bled 18 times from September 1994 to July 1995. All AGID and cELISA results were negative. Serological testing and virus isolation from the Shizong site are still in progress.

Discussion

Understanding the activity and distribution of bluetongue virus is very complex in Yunnan Province: as 94% of the area is mountainous, altitudes and climates vary from site to site. The selection of sites and animals for sentinel herds is important. It is essential first to survey animals in an area to determine sites where BLU is active, then to move seronegative animals, if necessary, from a place free of BLU antibody to the site where the sentinel herd is to be established. If the animals were born in the village where they will be used as sentinels, it is best to select and test animals aged 0.6–1 year: this minimises interference from maternal BLU antibodies and overcomes the difficulty of finding seronegative animals.

At the Kunming sentinel site, there were no BLU-positive animals among the 47 in the preliminary survey, and none of the 15 goats and 2 cattle showed seroconversion. This suggests there is no bluetongue virus activity around the area of Shuanglong Village in Kunming.

In Shizong, of the 94 animals in the preliminary survey, 37 were BLU-positive by cELISA and 22 positive by AGID. Serum neutralisation (SN) tests indicated the presence of BLU1 antibodies. A group of 12 sentinel cattle has been moved from Shuanglong Village, which is bluetongue-free, and serology and virus isolation will be performed to monitor activity at Shizong during the 1995 season of virus activity.

In Eshan county, 104 cattle and goat serum samples were collected from Eshan County in the preliminary survey: 44 animals were BLU-positive by cELISA and 29 positive by AGID. Neutralisation tests indicated the presence of BLU1, 15 and 16 antibodies. Of the 10 sentinel cattle, seven were BLU positive, making it difficult to detect infection by seroconversion. Future work will involve identification of BLU serotypes by neutralisation tests and isolation of virus from blood samples. An additional sentinel herd of 15 goats free from BLU antibodies has been introduced.

In summary, the serological results of a preliminary survey at Shuanglong (Kunming), Shizong and Eshan in Yunnan Province indicated that that there had been no BLU activity in recent years at Shuanglong (Kunming), and that Shizong and Eshan were suitable places for establishing bluetongue sentinel herds. Serology (by AGID, cELISA and SN) and virus isolation for the sentinel herds are still in progress. It is hoped to establish more sentinel herds in the southern counties of Yunnan Province with the collaboration of county veterinary stations. The stablishment of sentinel herds in other Provinces is also being discussed.

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Epidemiological Considerations in the Study of Bluetongue Viruses

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Abstract

Epidemiological studies of bluetongue viruses depend on understanding certain key features of the biology of the 24 serotypes. Bluetongue viruses are arboviruses, spread by insect vectors comprising some species in the genus *Culicoides*. Among ruminant hosts, cattle are the major reservoir or amplifier host, with only some breeds of sheep showing disease. Bluetongue viruses have both serotype and serogroup reactive antigens, and also show serological cross-reactions with other viruses. Serological tests must be interpreted on the basis of this knowledge. The distribution of bluetongue viruses can be mapped through serological surveys, but confirmation of virus presence in any area requires virus isolation. Monitoring sentinel groups of animals at frequent intervals allows both isolations and descriptions of seasonal patterns of seroconversions. Isolating viruses from insects allows identification of potential vectors, which must be confirmed by experimental transmission studies. Molecular virology allows comparison of sequence data from isolates, so that the relationships between viruses in different geographical areas can be studied. Pathogenicity does not depend on serotype but must be tested experimentally to determine the potential economic problem constituted by viruses from a given area. The financial cost to farmers, a major consideration in any epidemiological study, can only be determined by accurate on-farm records and accurate diagnosis of disease outbreaks. The application of these principles has been demonstrated in northern Australia and Indonesia, with good results. Much is now known of the distribution of the bluetongue virus group in these neighbouring countries, and eight bluetongue serotypes have been isolated from sentinel cattle in both countries.

BLUETONGUE viruses are arboviruses, so their epidemiology depends on their vectors, insects of the genus *Culicoides*. Epidemiological studies of arboviruses are incomplete without integrated vector studies (Daniels et al. 1991). Only a few species of *Culicoides* act as vectors, so to understand local influences on virus epidemiology each country or site must know which of its insect species are competent vectors; the relative infection rates of these species; and other aspects of insect biology .

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Bluetongue viruses form a serogroup in the genus *Orbivirus*, members of which have 10 double stranded RNA segments. Two structural proteins, VP3 and VP7, form the inner capsid carrying serogroup-specific antigens. The outer capsid comprises two structural proteins, VP2 and VP5, carrying serotype-specific antigens (Huismans and Erasmus 1981). The close antigenic relationships among members of the *Orbivirus* genus gives rise to serological cross-reactions among the antibodies to the serogroup antigens (Della-Porta et al. 1985). Serological studies to detect exposure to bluetongue group viruses should use tests designed to overcome this problem.

Within the bluetongue virus (BLU) serogroup, there are 24 serotypes, distinguished on the basis of serological neutralisation tests. However, detection of serotype-specific antibody in an animal does not prove infection with that serotype, since heterotypic

antibody responses may develop after exposures to two or more serotypes, resulting in the presence of neutralising serotypic antibodies in animals not exposed to these serotypes (Della-Porta et al. 1985). Confirmation of the presence of any BLU serotype at any geographic location should be based on isolation of the virus rather than on serological responses in exposed animals. Although ruminants are the mammalian hosts for bluetongue viruses, the patterns of infection and disease among these hosts are not understood completely.

Bluetongue Disease or Bluetongue Infection?

Epidemiologically, it is essential to distinguish between bluetongue disease and bluetongue infections. The epidemiology of the disease cannot be understood without understanding that infection only rarely results in disease. Conversely, the ecology of bluetongue virus infections cannot be understood if disease is used as a marker of infection. Two factors are of major importance. First, among ruminant hosts, there is considerable variation in species and breed susceptibility. Second, among the viruses there is considerable variation in strain pathogenicity that is independent of serotype.

For example, in Indonesia, bluetongue disease has been reported only in imported Suffolk sheep (Sudana and Malole 1982). Nevertheless, serological studies have shown cattle and buffaloes have higher prevalences of BLU infection than small ruminants, but show no associated disease (Sendow et al. 1986, 1991). Local (hair) sheep and goats show lower but significant prevalences of infection, but no disease has been reported in these breeds either.

This pattern, of high prevalences of infection but low or no occurrence of disease, has been recognised frequently in tropical and subtropical countries (Gibbs 1992). Breed susceptibility has been recognised as one factor, with introduced European breeds of sheep being more susceptible than local sheep breeds. Cattle are virtually non-susceptible to bluetongue disease, but are highly susceptible to BLU infection, with periods of viraemia lasting several weeks (MacLachlan et al. 1992; Melville and Hunt these Proceedings). Cattle are amplifier hosts of infection. Erasmus (1975a) considered a cattle–vector cycle adequate to maintain bluetongue viruses in the environment, and further suggested that sheep are merely accidental hosts.

Nonetheless, bluetongue is an Office International des Epizooties (OIE) List A pathogen because of the severe clinical disease, accompanied by high mortalities that does occur where susceptible breeds of sheep are infected with pathogenic virus strains (Erasmus 1975b). Hence, the purpose of any epidemiological study must be defined in terms of whether its subject is the disease or the infection. Clearly, studying the infection is necessary for any study of the disease, but often bluetongue infections are studied in the absence of any associated disease, frequently focussing on cattle, considered the main BLU host.

Basic Epidemiological Studies of Bluetongue Infection

Bluetongue virus infections are studied as one of the major risk factors associated with bluetongue disease. Where bluetongue disease is an existing or potential problem, knowledge is required of all aspects of BLU infections in both 'silent' and susceptible hosts, and in vectors. Many countries seek to protect their susceptible ruminant livestock through quarantine barriers, restricting movement of livestock and germplasm. Exporting countries thus need to have knowledge of their bluetongue infection status to support regional or global international trade. Ideally, importing countries will also clarify their own bluetongue infection status so as not to interrupt trade unnecessarily. Studies of bluetongue infections commonly include serological surveys, monitoring of sentinel herds, and vector studies, each of which is described in some detail below.

Serological (Cross-sectional) Surveys

In the epidemiology of bluetongue, serological surveys are useful primarily to define areas of risk, based on the usual infection status of the ruminants in the area. Countries may comprise zones of different infection status, and serological surveys help define the boundaries of these zones. Usually it is most important to identify areas where bluetongue infections are present or absent. In large countries, such as Australia, that have both infected and uninfected areas, there is little benefit in blending data, for example to give an overall prevalence of infection for that country. Rather, the information most useful to livestock producers and traders are the boundaries between zones where animals are possibly infected and zones where they are not. This allows assessment of the risk to livestock enterprises.

The risk of disease in susceptible sheep, or the risk of including a seropositive animal in a shipment for export, may be presumed to be higher where the incidence of infections is higher. Surveys therefore also may be used to define areas of high and low prevalence. In some countries or regions, such as northern Australia, some BLU serotypes are restricted to certain geographical areas. In such cases, serological

surveys have helped define the boundaries of the restricted area.

However, there are problems associated with cross-sectional serological surveys as an epidemiological technique. First, surveys give a single view of a changing situation. Second, the history of sampled animals is often not known with reliability. Third, some bluetongue serological tests are difficult to interpret because of cross reactive group antibodies and heterotypic antibodies (for example with the EHD serogroup). To be accurate, serological surveys for BLU group viral infections must use the cELISA test (Lunt et al. 1988) to avoid the problem of cross reactions experienced with other tests, such as the agar gel immunodiffusion (AGID) test (Della-Porta et al. 1985).

Bluetongue viruses are spread by vectors, the range of which may vary each year depending on the suitability of the climate for the vector's survival, especially at the edge of its range. This means that a survey conducted just once will give information only on virus distribution in the current or previous seasons. To monitor the distribution of a BLU infection over longer periods of time, serological surveys would have to be repeated at frequent intervals. Properly conducted surveys are expensive, both in the sampling phase and in the need to test an adequate number of samples for a defined level of confidence.

In many countries, livestock are highly mobile, being traded frequently. It may be uncertain whether an animal has spent its whole life at the location where it is being sampled: this in turn gives rise to doubt whether its antibody status accurately reflects BLU infections at that location. Similarly, there may be difficulties associated with the age of animals sampled in a survey. Older animals may have been exposed more often than younger animals to risks of infection, so results from a survey have to be corrected for age, which itself may be difficult to assess accurately. More frequent exposures, particularly to multiple serotypes, increase the probability of finding serotype-specific antibodies to serotypes other than those infecting the animal. Attempting differentiation between these heterotypic and 'real' responses can be difficult, and almost arbitrary. Serotype-specific information in which complete confidence can be placed may be unobtainable from a survey, particularly if older animals are sampled.

Serological surveys do have particular uses. A well planned and conducted survey, with a statistically valid sampling protocol (Cannon and Roe 1982), can give preliminary information about infections in livestock across a wide area, including susceptibility of different species to infection and probable boundaries between areas of different infection status. In these ways survey results can assist with the planning of other epidemiological investigations, such as the use of sentinel herds.

In Indonesia, for example, a preliminary serological survey showed that bluetongue infections were widely distributed on all the major islands, and that cattle and buffaloes had higher prevalences of infection than sheep and goats (Sendow et al. 1986, 1991). Cattle were subsequently chosen as sentinel animals (Sendow et al. 1992). In Australia, serological surveys have shown that bluetongue infections have a boundary contiguous with that of the most widely distributed *Culicoides* vector species (Della-Porta et al. 1983). Such surveys have also shown that, of eight BLU serotypes known in Australia, only two have spread from a northern focus to states on the eastern half of the continent (Ward et al. 1995). A more recent survey has defined further the boundaries of this northern focus of the remaining six restricted serotypes (Fig. 1; Daniels and Melville, unpublished data). Hence, the cross-sectional survey is useful for describing the patterns of exposure in the populations under investigation, information that can be used as the basis for further decisions.

Sentinel Herds

Sentinel herds (St. George 1980) are a form of structured surveillance, with a design analogous to the prospective, or cohort, study format. As such, they are one of the more powerful investigative strategies available to epidemiologists. Advantages of sentinel herd programs are those of prospective studies in general, as outlined in standard epidemiology textbooks. In particular:

- incidence rates can be estimated;
- the timing of infections can be observed;
- animals of known source and history of exposure can be sampled;
- management variables, such as insecticide use, can be controlled;
- program design can be flexible in terms of sampling frequency and choice of test;
- as the definitive test, the sampling design can accommodate attempted virus isolation, which is a more precise estimate of infection status than serology; and
- a flexible response to events can be a design feature; this may involve changing the sampling or testing procedure upon first observing infection in the group.

In bluetongue epidemiology, the primary purpose of a sentinel herd program, or of an individual sentinel group, is to detect BLU infections occurring at a particular place. Thus the identifying characteristic of the cohorts is their location. Evidence for BLU infec-

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In bluetongue epidemiology, the primary purpose of a sentinel herd program, or of an individual sentinel group, is to detect BLU infections occurring at a particular place. Thus the identifying characteristic of the cohorts is their location. Evidence for BLU infections at different locations can then be compared, as can the effect of secondary factors, such as climate, that may influence events at each location. To avoid confounding factors, sentinel groups should comprise animals of similar age and susceptibility to BLU infection. Based on the considerations outlined above and described by Erasmus (1975a), yearling cattle are the most appropriate sentinels of BLU infection, even though they will not show bluetongue disease. The only feature distinguishing groups of sentinels should be geographical location.

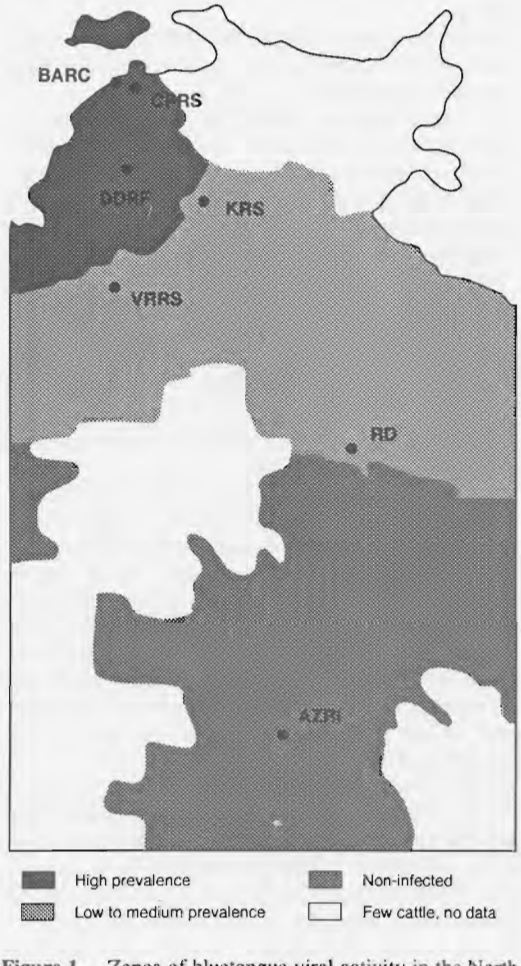

Figure 1. Zones of bluetongue viral activity in the Northern Territory of Australia, as defined by serological surveys and monitored by sentinel herds. Sentinel herd sites: BARC, Berrimah Agricultural Research Centre; CPRS, Coastal Plains Research Station; DDRF, Douglas Daly Research Farm; KRS, Katherine Research Station; VRRS, Victoria River Research Station; RD, Rockhampton Downs; AZRI, Arid Zone Research Institute.

Care is also necessary in choosing the actual site for the sentinel group. The aim is to maximise the chance of detecting viral activity at the geographical location for which the sentinel site acts as a sampling point. Alternatively stated, the purpose, particularly for monitoring, is to disprove, with as rigorous a challenge as possible, the null hypothesis that infections with bluetongue viruses do not occur in the geographical area under study. Hence the sentinel site should be in a local environment that is suitable for vector activity and adjacent to other mammalian hosts, where any infections in the area may be expected to spread readily.

Sera from surveys and sentinel herd programs should be stored methodically in a serum bank (St. George 1979; Young et al. 1985), which allows retrospective studies in the event of new viruses or serotypes being isolated, as described by St. George (1980) and Gard and Melville (1989).

Designing a sentinel program

The design of a sentinel herd program will reflect a compromise between the purposes and the resources available. This will affect the number of locations (sentinel sites) established, the frequency of sampling and the choice of test performed on the samples.

Virus isolation

The definitive measure of a region, country or zone's bluetongue infection status is the isolation of viruses and their identification to serotype. Clinical disease can identify an animal from which virus may be expected to be isolated but, as explained above, most BLU infections of livestock are asymptomatic. Experience has shown that the most efficient way to isolate arboviruses is to sample mammalian hosts prospectively at frequent intervals over the period when exposure may be expected, to ensure that a sample is collected during the viraemic period of each infection. This strategy has yielded isolates of multiple BLU serotypes in both Australia and Indonesia (St. George et al. 1980; Gard and Melville 1992; Daniels et al. 1996; Sendow et al. these Proceedings).

of embryonated chicken eggs (Gard et al. 1988). If resources are limited, weekly sampling may be restricted to that period of the year when vector and virus activity is considered most likely.

Monitoring

To detect changes in the distribution of monitored viruses beyond their normal range, sentinel groups may also be located on the usual boundaries of infected zones. In this case, an adequate observation may be the appropriate serological test, with the sampling frequency being one sufficient to detect a seroconversion in an appropriate time frame. For example, if monitoring is for trade support purposes (that is, to certify areas as infected or free), then sampling at the beginning and end of the expected vector season may be adequate. However, if the purpose is to detect movement of virus towards susceptible sheep, then more frequent sampling may be needed.

Description of seasonal patterns

An integral part of the epidemiological descriptions of arboviruses is knowledge of their seasonal patterns of infection, which usually result from climatic influences on vector populations. Monthly serological observations on sentinel groups can identify, with considerable accuracy, the temporal patterns at each location (Sendow et al. 1992), and possibly identify movements between locations.

For example, Australia has implemented a National Arbovirus Monitoring Program based on sentinel herds (Kirkland et al. these Proceedings) to confirm each year the infected or non-infected status of designated zones. In northern Australia, one of these sentinel herds is located in an infected area known by previous experience (Gard and Melville 1992), and confirmed by serological surveys, as the focus of infections with the full range of eight BLU serotypes known in Australia (Fig. 1). The site is sampled weekly for virus isolation, to detect any changes in the BLU strains present. Other sentinel groups, sampled monthly for serology, monitor the spread of usually restricted serotypes beyond their usual distribution, as defined by previous serological surveys (Fig. 1). Monitoring a southern sentinel site in the uninfected zone twice each year, before and after the summer, gives confidence that that zone remains uninfected. Thus the program comprises sentinel groups of cattle strategically placed for different purposes, and sampled and tested differently according to that purpose.

Vector Studies

Bluetongue viruses are spread by vectors. Movement of infected animals is relatively unimportant in the spread of infections, and movement of uninfected, seropositive animals appears of no consequence (St. George these Proceedings). Vector studies are an essential component of BLU epidemiological studies. The primary objective is to identify positively the vector species in each country or region, and establish those aspects of the vectors' biology that affect the patterns of virus spread.

Confirmation of vector status of an insect species is a multi-step process:

- the strength of the association between the vector species and its mammalian hosts must be considered;
- bluetongue viruses should be detectable in specimens of the suspect vector species that have been caught in the wild;
- the insect species should be shown to become infected through feeding on a source of virus; and
- most importantly, the species must be demonstrated to be able to transmit the virus biologically unless experimental work has established this last criterion, an insect species cannot be proven as a vector (Standfast et al. 1992).

Once the vector species in each country or region have been identified, further epidemiological information is needed for a full understanding of patterns of infection, or for modelling. This information includes the seasonal abundance of the vector species, and the infection rates of the vectors for the relevant arboviruses or serotypes.

Some *Culicoides* species breed in cattle faeces, and so may be distributed together with the main mammalian host to the limits of climate that are compatible with vector survival. Conversely, vector species with a restricted habitat do not pose a threat in spreading the virus beyond the confines of their habitat, even if they are highly efficient vectors. To be useful for either descriptive or predictive purposes, an epidemiological understanding of BLU infection must include knowledge of the factors limiting the distribution of each vector species.

Experimental studies of infection rates of vector species (Standfast et al. 1992) give an estimate of the probable efficiency of the vector in spreading bluetongue infections among mammalian hosts. Again, any conceptual model of the way bluetongue viruses survive in the environment, and of the way cycles of infection occur, will depend on such knowledge for each vector species. Where the important vector species are known, they can be monitored to detect and analyse changing risks of transmission of viral infections.

PCR detection of viruses in insects caught in the wild

Insects are frequently caught in light traps and stored in 70% alcohol before identification, allowing

samples to be obtained from remote areas (Sukarsih et al. 1993). However, such specimens are not suitable for subsequent virus isolation or for serologically-based antigen detection techniques. Amplification of viral nucleic acid by the polymerase chain reaction (PCR) offers a means of identifying BLU presence in such specimens (McColl and Gould 1991; McColl et al. 1994). Thus PCR provides a new technique for making observations in epidemiological studies.

As with any test system, correct application depends on standardisation of the test in each of the circumstances in which it may be applied. Primers controlling the PCR can be designed to be BLU group specific, or to allow detection of specific serotypes (McColl and Gould 1991). The sensitivity of each PCR must be established. Current work is investigating the sensitivity of the BLU group PCR in alcohol-fixed insect preparations (Melville and Hunt these Proceedings).

Two unique applications are being developed for PCR detection of bluetongue viruses in insects. In the first, observations on the cycle of BLU infections in sentinel cattle are being extended by examining the infections in *Culicoides* vector species occurring at the sentinel site at the same time. It will thus be possible to describe the complete cycle of virus infection through both vector and mammalian hosts. As described above, the infection status of sentinel cattle in northern Australia is determined regularly by virus isolation. Concomitant studies of PCR detection of BLU in insects sorted to species should allow determination of the vector species introducing the virus to the herd, and of the vector species propagating the wave of infection through the herd. The species still infected at the end of the infection cycle may have a role in maintaining virus in the environment, through virus spread to adjacent groups of uninfected mammalian hosts. Furthermore, comparisons can be made of the dynamics of infection cycles of different BLU serotypes, to attempt to explain the different patterns of spread observed among serotypes (Fig. 1).

After the sensitivity of PCR detection of virus in alcohol-fixed insects has been established with confidence, PCR should be a useful monitoring tool in locations where the sampling of mammalian hosts is not practical. PCR testing of pools of insects from remote areas may allow detection of bluetongue viruses in such locations, if needed for monitoring or during the response to emerging situations identified by routine monitoring. Since the only aim would be to detect virus, not to identify potential vectors, it would not be essential to identify insects to species in a routine monitoring situation. However, use of PCR would be more focussed, and hence more efficient, if just pools of known vectors were processed. These applications depend on existing knowledge for their usefulness. In northern Australia, experimental studies have already identified the vector species (Standfast et al. 1992), and an efficiently functioning sentinel herd program provides the framework for studies of virus dynamics. Thus PCR studies are seen as extending, not replacing, more conventional procedures.

Molecular epidemiology and topotyping

Molecular epidemiology may be defined as the study of the relationship of an organism to other organisms on the basis of genomic analyses, for the purposes of recognising spatial and/or temporal differences or changes in populations.

Differences can be detected in the genomes of apparently similar viruses isolated from different geographical regions. For example, RNA–RNA hybridisation studies showed differences between BLU isolates from Australia and Africa (Huismans and Bremer 1981; Gorman et al. 1981). Similar results were obtained with RNA–recombinant DNA hybridisation (Gould 1988). Nucleotide sequencing of the gene segments being compared brought considerable analytical capability to these studies, and comparison of nucleotide sequences confirmed the differences among BLU isolates from different geographic regions (Gould 1987).

Similar studies have been conducted with other arboviruses. Isolates of St. Louis encephalitis virus were compared on the basis of oligonucleotide fingerprints: isolates could be grouped on the basis of similar fingerprints, and the different groups originated from different geographical areas within the USA. The term 'topotype' was used to refer to these groupings (Trent et al. 1981). Similar approaches have led to the description of topotypes of dengue virus serotype two in South East Asia (Trent et al. 1989).

Sequence analysis of BLU isolates, and comparison with known groups defined on the basis of sequence homology, again offers a new observational strategy for epidemiological studies. If the normal BLU 'topotype' has been determined at any location, the sequencing of new isolates will help confirm the stability of the virus–host ecosystem in that place, or, alternatively, detect new virus incursions into the area. If the sequence data of the new incursion show homology with another known grouping, an inference may be made about the possible source of the new viral incursion. Even if the new incursion is of a serotype that already occurs in the area, the difference in nucleotide sequence gives a marker for monitoring the spread of the introduced virus. This could have important practical benefits, for the introduced virus may be more pathogenic than existing strains, and hence be associated with an increased risk of disease in susceptible sheep.

For example, the Australian prototype isolates of the eight BLU serotypes so far identified may be grouped into two topotypes on the basis of sequence analysis of their viral RNA segment 3, with isolates within groups showing more than 95% sequence homology. Seven isolates, of BLU1, 3, 9, 16, 20, 21 and 23, group in one topotype while the prototype isolate of BLU15 has considerably less homology with the other isolates, and so must be considered in a different grouping (Gould 1987; Gould and Pritchard 1991). However, the sequence data of more recent Australian isolates identified viruses that did not group with those originally described (McColl et al. 1994; Melville and Hunt these Proceedings). Further research is needed to determine whether these newer isolates have sequence homology with viruses from other geographic regions, and whether they may be new incursions from such regions.

Basic Epidemiological Studies of Bluetongue Disease

The approaches discussed above are applicable to the epidemiological study of bluetongue infections irrespective of whether such infections are associated with disease. The diagnosis of bluetongue disease in a country necessitates an extra dimension to epidemiological studies, to describe the impact of the disease on the susceptible sheep population. There is a need to ascertain the true incidence of disease morbidity and mortality, so that an assessment may be made of the economic cost to farmers and to the nation. Only on this basis will it be possible to estimate the cost-effectiveness of control measures.

There are three broad approaches to data collection, and the strengths and disadvantages of each have been evaluated critically by Daniels et al. (1993). The appropriate mix of approaches will depend on the veterinary infrastructure of the affected country, on the size of farms and on the social organisation of the farming systems. Assessing disease-associated losses in systems based on smallholder farmers living in villages, on nomadic herdsmen or on extensive pastoral holdings will require different adaptations of the basic principles.

Routine (passive) data may be available from veterinary field reports or laboratory records. However, such data often suffer from major biases associated with variables in reporting and specimen submission (Martin 1993). As with studies of infections, the most accurate data on disease occurrence will result from programs of structured surveillance (Morris and Leidl 1993), in which there is active monitoring for cases accompanied by accurate laboratory diagnosis. Such studies require considerable management inputs. An alternative approach is to attempt to compile farmers' local knowledge through various strategies of participative appraisal (Young 1993): this requires development of a consensus between investigators and farmers of an appropriate clinical case definition for use in on-farm interviews.

Another aspect of the study of bluetongue disease is confirmation of the relative pathogenicity of the bluetongue viruses present, through experimental infections of susceptible sheep. Virus pathogenicity is independent of serotype: BLU1 in South Africa is highly pathogenic (Gard 1987) while the first BLU1 in Australia is only mildly pathogenic (Hooper et al. 1996). To give confidence to the interpretation of epidemiological studies, pathogenicity should be confirmed experimentally. Although the marker or feature conferring pathogenicity to bluetongue viruses is not known, observations show that these viruses become attenuated after passage through cell culture (Gard 1987). Inocula for experimental studies must be viruses passaged only in ruminants. Comparative studies of viral pathogenicity should also standardise the age and breed of experimental sheep (Johnson et al. 1992; Sendow et al. these Proceedings).

A Regional Perspective

Bluetongue viruses are vector-borne, and so cannot be contained or excluded by national boundaries, even when these are supported by efficient quarantine services. The epidemiology of bluetongue infections can be more easily understood with collaboration between neighbouring countries, or among countries within a geographical region. The sharing of technology and data will allow each participating country more fully to appreciate influences affecting its current situation, and challenges that may arise in the future.

Indonesia and Australia have developed such collaboration over several years (Daniels et al. 1996). Close similarities in the BLU fauna of both have been identified, with six serotypes BLU 1, 3, 9, 16, 21 and 23 occurring in each country (Melville and Hunt 1996; Sendow et al. these Proceedings). BLU7 and BLU12 have been isolated in Indonesia but not in northern Australia, while BLU15 and BLU20 have been isolated in northern Australia but not in Indonesia (Gard and Melville 1989). However, serological studies (Sendow et al. 1991) do show evidence of BLU20 in Indonesia. Further north, BLU1, 3, 9, 16 and 23 have also been isolated in Malaysia, where an additional serotype, BLU2, has also been reported (Sharifah et al. 1995). Such comparisons raise questions about whether the serotypes apparently restricted to one country at present will be found to be

more widely dispersed, and whether the viruses apparently common to two or more countries comprise part of more broadly distributed populations or whether they are separate foci of infection.

An important consideration in any region is the pathogenicity to sheep of the region's local and nearby bluetongue viruses. In the Southeast Asian and Australian region, naturally occurring bluetongue disease has been reported in both Indonesia and Malaysia in imported sheep, but not in local sheep also exposed to infection. In Australia, susceptible sheep are not usually exposed to BLU infections, being reared in the BLU-free zone. Pathogenicity tests in Indonesia (Sendow et al. these Proceedings) and Australia (Hooper et al. 1996) have shown that the strains tested in each country are of relatively low pathogenicity. Countries in this region should monitor for the introduction of more pathogenic strains.

Molecular epidemiological techniques, as described above, appear to offer a means of monitoring the distribution and movement of BLU strains within a region. For example, initially a definite regional grouping of Australian bluetongue viruses was observed (Gould 1987; Gould and Pritchard 1991; Pritchard et al. 1995). However, the latest observations show that some more recent isolates in northern Australia are genetically distinct from the prototype isolates (Melville and Hunt these Proceedings). Indonesian BLU isolates are distinct from the original Australian grouping, and furthermore do not comprise an homogenous group (Sendow et al. these Proceedings). Further analysis of the data from a regional perspective will attempt to identify broader groupings of isolates based on the degree of closeness of genetic relationships. Probable patterns of movement of virus strains may be identified, as may changes in the genetic composition of virus strains over time or in response to other epidemiological influences.

Conclusion

Bluetongue viruses can cause severe disease in some sheep, but usually do not do so, nor do they cause disease in cattle, the main mammalian host. Bluetongue viruses are widely distributed in tropical and subtropical countries, frequently occurring as inapparent infections. Epidemiological investigations based on sentinel herd techniques allow isolations and other studies of all BLU infections, not just those associated with disease, and form the basis for further analyses of virus biology and epidemiology. Where bluetongue disease occurs, serious financial losses have been reported. Molecular studies show that strains of these vector-borne viruses can be highly mobile between geographical locations. Effective monitoring for changes that may be associated with a higher risk of disease requires isolation and characterisation of bluetongue viruses.

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Entomology

Introduction

THE epidemiology of an insect-borne virus disease such as bluetongue cannot be understood without a knowledge of its insect vectors. If we are to understand the dynamics of transmission, we must know which *Culicoides* species transmit the virus, their relationship to the vertebrate host and the efficiency with which different species transmit the different serotypes.

There is a similarity between the present situation in China and the situation in Australia in 1977 when bluetongue was first discovered there. In China, there are at least 73 known species of *Culicoides*, possibly a third of which feed on sheep and may be involved in the transmission of bluetongue virus. In Australia, at least 150 *Culicoides* species are known. These figures suggest that there are many more species in China waiting to be discovered. In Australia, we did not know which species transmitted the virus but suspected *C. marksii*, which was known to feed on sheep and from which 40 isolations of an orbivirus had been made. However, when this species was tested by feeding it on infected sheep it did not become infected with bluetongue virus. However, several other *Culicoides* species were capable of being infected in this way.

So one lesson to be drawn from the Australian experience is that all species of *Culicoides* that feed on sheep and cattle must be tested for ability to transmit the virus. Another lesson is that the species of *Culicoides* able to be infected had different susceptibilities to different serotypes, and individual species transmitted different serotypes with varying efficiency.

Hence, the emphasis of the Symposium's Entomology Workshop was on simple techniques to answer the question 'What species of *Culicoides* transmit bluetongue, and how efficiently do they transmit it?'

Culicoides Survey in Indonesia

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Abstract

Culicoides were collected by light traps at sites adjacent to livestock, especially penned cattle. The areas surveyed were West Java (Depok and Cisarua), Bali, Nusa Tenggara Timur (Kupang) and Irian Jaya (Jayapura and Merauke). The survey sought to identify *Culicoides* species found in those areas and to isolate bluetongue virus from *Culicoides*. The most abundant species in Irian Jaya and Bali was *C. peregrinus*; in Nusa Tenggara Timur *C. oxystoma*; in Cisarua *C. parahumeralis*; and in Depok *C. sumatrae*. *Culicoides brevitarsis* and *C. fulvus*, species reported as vectors of bluetongue virus, were found at all surveyed locations, while another vector, *C. actoni*, was found in Irian Jaya and Java but not in Kupang or Bali. *Culicoides orientalis*, which is closely related to vector species, was also found at all locations. Bluetongue virus (BLU) serotype 21 was isolated from a pool of *C. fulvus* and *C. orientalis*.

MANY haematophagous insects are of economic importance because they transmit disease. Kettle (1984) reported the importance of *Culicoides* midges in the transmission of disease organisms to humans and animals. Among the more important viruses for which *Culicoides* are vectors is the bluetongue virus (BLU), which has a global distribution involving Africa, Asia, Australia and North and Central America. Epidemiological observations have shown that, although bluetongue infections are endemic in tropical areas of the world such as Indonesia, there is no associated disease in populations of local sheep (Sendow et al. 1989, 1992). However, as bluetongue disease has been reported in imported sheep in Indonesia (Sudana and Malole 1982), the virus is important in the context of its potential impacts on trade and development programs.

Implication of any insect species as an arbovirus vector is a multistep process. The insect must be known to feed on the mammalian host and the virus must be isolated from the insect in nature. Experimental studies should also show that the insect is able to transmit the virus biologically among mammalian hosts. In Indonesia BLU serotype 21 has been isolated from a mixed pool of *C. fulvus* and *C. orientalis* of the subgenus *Avaritia* (Sendow et al. 1993). The transmission of bluetongue viruses by *C. fulvus, C. wadai, C. actoni,* and *C. brevitarsis* has been reported in Australia, which neighbours Indonesia (Standfast et al. 1985). These species may therefore be considered potential vectors in Indonesia.

As a first step in determining bluetongue vectors in Indonesia, surveys have determined the range of *Culicoides* species present in major livestock-producing areas. While some results have been presented previously (Sukarsih et al. 1993), this paper presents additional data on the *Culicoides* species found in some areas of Indonesia, and recommends further avenues for investigation.

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Materials and Methods

Collection sites

Collections were made adjacent to sentinel groups of cattle which were monitored separately for serology and virus isolation (Sendow et al. 1989, 1992). These sentinel groups were at sites located strategically across the length of Indonesia, including West Java (Depok and Cisarua), Bali (Denpasar), Nusa Tenggara Timur province (Kupang on West Timor, and Rote Island) and Irian Jaya (Jayapura, Merauke, Wamena, Timika and Biak). Other collections were made from some farming areas in Central Java (Semarang), Lampung province and South Kalimantan (Banjarbaru).

Collection and processing procedures

In Bali, Lampung and Banjarbaru, battery-powered, modified Centers for Disease Control (CDC) light traps were used, whereas in West Timor, Rote and Irian Jaya battery-powered, modified CDC light traps and Pirbright-type miniature light traps were used. The traps were sited adjacent to livestock, especially penned cattle, and also adjacent to standing surface water where possible. Traps were set in the late afternoon and allowed to run until daybreak. In West Java and Central Java insects were collected with Pirbright-type miniature light traps operated from the mains supply via a 12 volt step-down transformer. Collections were made from 4.30 pm to 8.00 pm. At sentinel sites collections were made once a week throughout the year, while at Wamena, Timika, Biak and Rote Island they were made monthly. At Lampung, Central Java and Banjarbaru collections were made during a single sampling expedition. The catches were held in 70% alcohol, except in West Java where, since virus isolation was to be attempted, the insects were blown directly into a bottle containing phosphate buffered saline (PBS) supplemented with antibiotics and containing 0.1% detergent.

The catches from each location were sorted in the laboratory under a binocular microscope. From catches in West Java, the female *Culicoides* were sorted to species and parous females separated on the basis of abdominal pigmentation (parous adults were distinguished by the development of dark red pigment in the epidermal or subepidermal layers of the abdomen; Dyce 1969). *Culicoides* identification was based on standard texts (Dyce unpublished; Ratanaworabhan 1975; Tokunaga 1959; Wirth and Hubert 1989). Numbers of each species for every trapping were recorded.

Insects difficult to identify were mounted for independent confirmation by A.L. Dyce. Mounted specimens were prepared by serial washing in graded alcohols (70%, 80%, 90% and absolute) followed by clearing in creosote, and mounted in thin xylol-balsam.

Results

Lampung (Sumatra)

Culicoides were collected from three locations in Lampung, with nine species recorded (Table 1). The dominant species was *C. orientalis*, followed by *C. sumatrae* and *C. peregrinus*. Vector species *C. actoni* and *C. fulvus* were also present.

Table 1. Species of *Culicoides* collected in Lampung, Banjarbaru and Central Java.

Species	Lampung	Banjarbaru	Central Java
C. actoni	28	32	7
C. arakawae	30	5	4
C. barnetti	10	3	10
C. fulvus	7	9	2
C. geminus	29	9	1
C. guttifer	10	1	5
C. orientalis	74	95	58
C. oxystoma	11	57	86
C. parahumeralis	29	5	10
C. peregrinus	39	123	180
C. sumatrae	51	76	3

Banjarbaru (South Kalimantan)

Eleven species were collected from two locations in Banjarbaru (Table 1). The dominant species was *C. peregrinus* followed by *C. orientalis* and *C. sumatrae.* Vector species *C. actoni* and *C. fulvus* were again present.

Central Java

Two locations at Semarang were sampled, yielding nine species. The most abundant was *C. peregrinus*, followed by *C. oxystoma* (Table 1). Again *C. actoni* and *C. fulvus* were the only recognised vector species present.

West Java

Culicoides collected at sentinel sites included 24 species at Depok and 22 species at Cisarua (Table 2). At Depok, *C. actoni* was the dominant species, followed by *C. parahumeralis, C. sumatrae, C. fulvus* and others. At Cisarua, *C. parahumeralis* was the dominant species, followed by *C. maculatus* and *C. orientalis*. The vector species *C. wadai* was present at Depok.

Bali

At Denpasar 17 *Culicoides* species were collected (Table 3). By far the most abundant was *C. peregri-*

nus, followed by *C. oxystoma, C. arakawae, C. brevitarsis and C. fulvus.*

Kupang, West Timor

At Kupang 23 species were collected (Table 3). *Culicoides oxystoma* predominated, with *C. brevitarsis, C. histrio, C. peregrinus* and *C. geminus* also well represented in collections, and *C. fulvus* and *C. wadai* present in smaller numbers.

Table 2. Species of *Culicoides* collected in Depok and Cisarua, West Java from October 1992 to September 1993.

Species	Depok	Cisarua
C. actoni	5870	16
C. anophelis	24	–
C. arakawae	8	–
C. barnetti	92	4
C. fulvus	1401	139
C. geminus	41	1
C. gewertzi	18	1
C. guttifer	21	4
C. huffi	16	3
C. insignipennis	40	90
C. jacobsoni	18	76
C. liui	1	57
C. maculatus	14	375
C. orientalis	809	154
C. oxystoma	681	9
C. palpifer	1173	125
C. parahumeralis	4523	838
C. peregrinus	957	11
C. shortii	371	1
C. sumatrae	2091	132
C. wadai	3	–
Total	18172	2036

Rote Island

During this survey on Rote Island, *C. brevitarsis* was the most abundant species among the 11 species collected (Table 3), followed by *C. oxystoma, C. peregrinus* and *C. wadai. Culicoides fulvus* was also present.

Irian Jaya

At the sentinel cattle site in Jayapura, 30 species of *Culicoides* were collected (Table 4), with *C. peregrinus* the most abundant and *C. brevitarsis* the second most abundant. All four known vector species were present. In Merauke, *C. peregrinus* was again the most abundant among the 20 species collected (Table 4), followed by *C. brevitarsis, C. orientalis* and *C. histrio*, with all four vectors present. At the remaining Irian Jaya sites fewer insects were trapped. Among the 13 species collected at Biak, *C. peregrinus* was predominant, with *C. brevitarsis* and *C. fulvus* also present. (Table 4). Of the six species collected at Wamena, *C. maculatus* was the most abundant, followed by *C. peregrinus* (Table 4), and *C. wadai* was also present. At Timika 12 *Culicoides* species were collected (Table 4) including *C. brevitarsis, C. fulvus* and *C. wadai.*

Table 3. Species of *Culicoides* collected in Denpasar, Kupang and Rote Island in South East Indonesia.

Species	Denpasar	Kupang	Rote Island
C. albibasis	–	7	–
C. anophelis	12	–	–
C. arakawae	73	33	8
C. barnetti	9	23	28
C. brevipalpis	–	20	–
C. brevitarsis	51	936	189
C. clavipalpis	4	–	–
C. effusus	5	31	–
C. flavescens	–	5	–
C. fulvus	26	38	19
C. geminus	7	409	–
C. gewertzi	–	12	–
C. guttifer	17	19	–
C. histrio	–	739	–
C. huffi	19	–	–
C. nudipalpis	11	3	3
C. orientalis	23	26	–
C. ornatus	–	–	2
C. oxystoma	97	2983	95
C. palpifer	5	8	–
C. pangkorensis	–	19	–
C. papuensis	–	7	–
C. parahumeralis	14	17	–
C. pelilioensis	–	3	–
C. peregrinus	1675	642	67
C. semicircum	5	–	–
C. sumatrae	–	45	10
C. wadai	–	33	59

Table 4. Species of *Culicoides* collected in Irian Jaya: Jayapura, Merauke, Biak, Wamena and Timika.

Species	Jayapura	Merauke	Biak	Wamena	Timika
C. actoni	12	9	–	–	–
C. arakawae	–	–	–	–	–
C. ardleyi	65	–	–	–	–
C. austropalpalis	2	5	–	–	–
C. barnetti	21	–	8	–	–
C. brevitarsis	895	215	10	–	9
C. dumdumi	4	–	–	–	–
C. effusus	14	–	3	–	5
C. fulvus	56	21	12	–	11
C. gemellus	8	12	–	–	–
C. geminus	–	–	–	–	–
C. gewertzi	31	–	–	–	–
C. guttifer	38	–	4	–	12
C. histrio	27	–	5	–	–
C. huberti	18	–	–	–	–
C. jacobsoni	15	37	–	–	–
C. maculatus	9	8	8	17	–
C. neomelanesiae	58	–	–	–	–
C. nudipalpis	19	2	–	–	2
C. orientalis	48	67	–	4	18
C. ornatus	2	7	–	–	–
C. oxystoma	7	98	13	6	27
C. palpifer	33	–	6	–	8
C. pampangensis	–	–	–	–	–
C. pangkorensis	174	5	–	–	–
C. parabarnetti	19	–	–	–	–
C. parabubalus	5	–	–	–	–
C. parahumeralis	42	–	23	–	41
C. peliliouensis	–	11	3	–	–
C. peregrinus	2013	1002	–	15	103
C. petersi	2	–	–	–	–
C. pseudostigmatus	4	9	–	2	–
C. pungens	–	3	–	–	–
C. semicircum	7	6	1	–	–
C. sumatrae	5	12	45	–	13
C. wadai	83	36	–	6	21

Discussion

This paper reports on a component of a major longitudinal study of *Culicoides* species in Indonesia, previous results of which have been reported by Sukarsih et al. (1993). As the present results complement the earlier ones, without duplication, they offer an opportunity to identify trends. In the absence to date of any specific vector competence work in Indonesia, attention has focused on species shown to be vectors in neighbouring Australia (Standfast et al. 1985).

Sukarsih et al. (1993) reported on collections from Depok, Cisarua, Denpasar, Kupang, Jayapura and Merauke. Two of the four known vector species, *C. fulvus* and *C. brevitarsis*, were identified at all sites in that study; *C. wadai* was absent from only one site (Denpasar); and *C. actoni* was only absent from Kupang and Denpasar. Sukarsih et al. (1993) thus

noted that all four vector species were distributed widely in both western and eastern Indonesia.

Also in the first period of the study, *C. brevitarsis* had been among the more abundant species in the east, but not in the west (Sukarsih et al. 1993). An unexpected finding in this second period was the failure to identify *C. brevitarsis* at a majority of sites in western Indonesia, including all three survey sites in Sumatra, South Kalimantan and Central Java, and the regularly monitored sentinel sites at Depok and Cisarua in West Java. However, *C. brevitarsis* was detected at most sites from Bali eastward, with the exception of the high altitude site at Wamena, and was the second most abundant species trapped at Jayapura, Merauke and Kupang, confirming a trend. (Although numbers of *Culicoides* in light trap collections need not necessarily correlate with the size of the natural population at sampling sites, such data are the only basis currently available in Indonesia on which to compare the abundance of the various species.)

During both survey periods, *C. fulvus* was abundant in collections from Depok and Cisarua, and was widely distributed throughout Indonesia. As the species with the highest identified infection rates for BLU viruses in Australia (Standfast et al. 1985), *C. fulvus* is viewed as a most important vector in that country (Daniels and Melville 1996). We strongly recommend further studies of the role of this species as a vector.

Sukarsih et al. (1993) reported that, although *C. actoni* was abundant at Depok, it was not among the ten most abundant species at any of the collection sites in eastern Indonesia. The present results confirm those observations, with *C. actoni* again being the most abundant species at Depok, but not represented in collections from Denpasar and Kupang, and with only a few individuals trapped in Irian Jaya. This species was present at each of the sites surveyed in Sumatra, Kalimantan and Central Java, confirming a wide distribution. The breeding site for this species is associated with vegetation (Wirth and Hubert 1989), a factor which may be associated with apparently low numbers in some areas.

In the first survey period, *C. wadai* appeared not to be present in large numbers, and was more frequently identified in Irian Jaya than elsewhere (Sukarsih et al. 1993). During the second period, only three insects of this species were trapped in western Indonesia, at Depok.

As the four vector species are all of the subgenus *Avaritia* (Wirth and Hubert 1989), Sukarsih et al. (1993) drew attention to the relative abundance and distribution of other members of this subgenus that should be tested for vector competence in Indonesia. *Culicoides brevipalpis* supports BLU growth, as do a range of other *Culicoides* species (Standfast et al. 1985), but has not been studied sufficiently to confirm or deny its vector status, and has been identified in Indonesia only in low numbers. *Culicoides orientalis* and *C. nudipalpis* both feed on cattle and have been trapped in relative abundance, particularly in the case of *C. orientalis* in western Indonesia. As *C. orientalis* comprised 20% of a pool of *Avaritia* subgenus midges from which BLU21 was isolated in West Java (Sendow et al. 1993), more specific studies of the vector potential of this species are considered important.

In both this survey period and that reported by Sukarsih et al. (1993), considerable numbers of *C. fulvus* were identified at Cisarua, where the species was respectively the second and fourth most abundant *Culicoides* in collections during the two reporting periods. Cisarua is a high altitude site 1300 m above sea level in West Java, some 75 km or more inland, with an average annual rainfall of 3500 mm. In Australia, *C. fulvus* has been trapped mostly in coastal areas in the far north (Johnson 1992). The larval habitat of *C. fulvus* is unknown (Wirth and Hubert 1989) although it is probably an important vector species, as suggested by its high experimental infection rates (Standfast et al. 1985) and its wide distribution and abundance in both Indonesia and northern Australia at sites with a high incidence of BLU infections (Daniels and Melville 1996). Further studies to identify the breeding and other habitat requirements of this species seem essential to a full understanding of bluetongue epidemiology in the Southeast Asian and Australian region.

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Preliminary Results of Trapping for *Culicoides* in South China: Future Bluetongue Vector Studies

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Abstract

Since the first isolation of bluetongue virus in China, clinical disease has been seen in four provinces, and bluetongue antibodies reported in sheep and cattle in 29 provinces. Biting midges, *Culicoides* spp., are the recognised vectors of bluetongue, and at least 30 species have been reported from southern China. However, little is known about which species feed on livestock or which may be the important vectors of bluetongue or other livestock arboviruses. This paper reports on the *Culicoides* species taken in preliminary surveys with light traps near livestock in Yunnan and Sichuan Provinces. Future plans are suggested, based on experience with bluetongue epidemiology in Australia. Bluetongue vector studies in China will need to use bait collections, and further light trap and, if possible, truck trap collections. These studies should follow a regular collection routine to show seasonality and abundance of different species. Potential breeding sites, particularly cattle dung, can be examined to determine basic associations. In the longer term, species found to be closely associated with livestock can be tested in the laboratory for their vector competence with bluetongue viruses. This would include feeding on virus either in vivo or in vitro. Suspected vector species can then be inoculated and tested for their capacity to transmit virus to animals or to excrete virus, using *in vitro* techniques. Knowledge of vectors is required for any attempt to control or reduce incidence of bluetongue disease. These studies should determine which species are the important vectors of bluetongue viruses in China.

CULICOIDES biting midges are small flies found in tropical to temperate regions on every continent. Many species have reputations as pests because of their highly irritating bite. However, they have a potentially more damaging role as they can carry and transmit several viruses associated with livestock. The most important of these are the bluetongue viruses (BLU). These infect ruminants and can cause severe disease and mortality in sheep, thus disrupting international trade in ruminants and their germplasm.

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Bluetongue was first recognised in China in 1979 (Zhang Nianzu et al. 1989), and, on the basis of clinical disease and/or antibody assessments, has since been reported in many of China's provinces and autonomous regions.

The recognition of bluetongue virus in China has focused attention on the *Culicoides* species of China. This paper provides some background on those species, and examines relevant vector competence studies in other countries. We also suggest how to proceed with such studies in China.

There are 24 BLU serotypes recognised around the world, with a small number of known vectors and many other potential vectors. Wirth and Dyce (1985) grouped enzootic bluetongue areas into three north–south zones, each with its own set of vectors cycling viruses which themselves seemed to fall into distinct groups. These three zones were Africa and the Mediterranean; Australia and eastern Asia; and the Americas. The most important BLU vectors in each zone

tend to be part of species groups with close taxonomic relatives that are difficult to separate.

In Africa and the Mediterranean, *C. pallidipennis*, a member of the subgenus *Avaritia*, was originally described as the vector of bluetongue. It was later realised that this species was a synonym of *C. imicola*, which itself can be readily confused with several species (Meiswinkel 1989). Similar problems exist with the *schultzei* group, another batch of potential bluetongue vectors. This species name was used by early workers but '*C. schultzei*' it is now recognised as a mixture of species.

In Australia and Southeast Asia, the taxonomic problems are similar to those in Africa, in that the subgenus *Avaritia* and the *schultzei* group are involved. However, the members of these groups are different from their African counterparts. In this region, the most common species in the *schultzei* group is *C. oxystoma*, and reference is often made to the *schultzei/oxystoma* group. In Australia, the unmasking of the species that could be infected with BLU was only possible because of detailed taxonomic studies by Alan Dyce (Dyce and Standfast 1979; Dyce 1982, 1989). Before these studies, all four potential vectors in the subgenus *Avaritia* were lumped together as *C. brevitarsis*. Subsequently, the original *C. brevitarsis* was identified as including *C. actoni, C. fulvus* and *C. wadai*. These four species have such different life cycles and vector competence rates for bluetongue that understanding the epidemiology of the virus in Australia would have been impossible without differentiating among these species. The subgenus *Avaritia* contains many very similar species across the Australasian and Oriental regions, and published descriptions could best be described as having been in a state of chaos until Alan Dyce was able to unravel much of the confusion (Dyce 1979, 1980, 1983; Dyce and Wirth 1983).

In the Americas, the taxonomic problems revolve around different *Culicoides* species. Identified as a BLU vector in the 1950s, *C. variipennis* is now considered to be a complex of subspecies of varying vector competence (Wirth and Morris 1985; Tabachnik 1992). A member of the subgenus *Monoculicoides*, '*C. variipennis*' has close relatives in Africa, Europe and Asia. None of these relatives have yet been implicated in natural BLU transmission, although some have been infected experimentally (Jennings and Mellor 1988). In Florida, BLU has been isolated from *C. insignis*, in areas where the virus is active in cattle but *C. variipennis* is not found (Greiner et al. 1985). Now confirmed as a vector in Florida, *C. insignis* is probably also a vector in the Caribbean and in South America (Tanya et al. 1992).

Clearly accurate taxonomy of vectors is an absolute prerequisite for unravelling the epidemiology of an arbovirus. Morphologically similar species and members of the same subgenus commonly have very different vector competences. Perhaps the best example is the group of four Australian BLU vectors *C. actoni, C. brevitarsis, C. fulvus* and *C. wadai*: though extremely similar in appearance, all have very different distributions, breeding sites and vector competence for BLU (Standfast et al. 1985).

Also in any epidemiological studies it is essential to keep good quality specimens, properly prepared and preserved. In the case of *Culicoides*, this means slide-mounted material. One of the problems with unravelling the *schultzei/oxystoma* group in particular is the loss, or poor quality, of type specimens available for study. Techniques for mounting small flies have been described (Steffan 1983; Wirth and Marston 1968): other unpublished methods are available from the authors. Producing good slide-mounted material is a combination of art and skill and requires much practice.

The *Culicoides* species reported as being present in China, and any known host associations of those species, are a starting point to which further information can be added (Table 1). Further synonyms may need to be determined, as well as some rearrangement of species names (Table 2). For example, one species is reported as *C. marginalis* Chu and Liu (Table 1), but this species must be renamed as the name already exists for an Australian species described by Lee and Reye (1962). Standfast et al. (1992) tabulated synonyms and confusion for three potential bluetongue vectors in the Oriental region (Table 3).

How do we approach these vector studies? Some of this work, such as that by Dr Weihan Zhou and his group in Anhui, is described elsewhere in these Proceedings. In Kunming, insect collections are being made. In both these places the importance of entomology studies is understood. However it is useful to look at our experience in Australia to make some pertinent points about studies of BLU vectors.

Field Investigations

The first step is to determine which *Culicoides* species are associated with, and feed on, ruminants in areas where bluetongue virus is active, and to investigate the activity patterns of these species. Several techniques can be used for this, each with advantages and disadvantages.

Light traps and truck traps

Light traps have many advantages. They do not require intensive labour, as traps can be set adjacent to livestock and left overnight. They can be set and cleared by operators who have had only minimal training.

Table 1. *Culicoides* species reported as being present in China.

Species	Reference	Host associations
C. actoni Smith	2, 4, 5, 6	human, livestock, marsupials
C. albifascia Tokunaga	4	
C. alexandrae Dzhafarov	7	
C. amamiensis Tokunaga (= *sumatrae* Macfie)	2, 3, 4	human
C. anophelis Edwards	2, 3	mosquitoes
C. arakawae (Arakawa)	2, 3, 5, 6	birds
C. aterinervis Tokunaga	1, 2, 4	
C. buckleyi Macfie (= *jacobsoni* Macfie)	4	
C. chiopterus Meigen	4	cattle, horse (dung breeder)
C. circumscriptus Kieffer	2, 3, 4	
C. caucasicus	7	
C. clavipalpis Mukerji	2	
C. crairi Kono and Takahashi	2	
C. dubius Arnaud	2	
C. duodenarius Kieffer	2	
C. elbeli Wirth and Hubert	3	
C. elongatus Chu and Liu	3	
C. flavescens Macfie	2, 3	
C. flaviscutatus Wirth and Hubert	2, 4	
C. fukienensis Chen and Tsai	2	human
C. fulvus Sen and Das Gupta	8	cattle
C. gemellus Macfie	2	
C. grisescens Edwards	4	
C. hamiensis	7	
C. homotomus Kieffer	2, 3, 4, 5, 6	human, cattle, buffalo, pig, sheep
C. hui Wirth and Hubert	2, 3	
C. humeralis Okada	2, 3, 4	
C. imicola Kieffer	8	cattle, sheep, horse
C. jacobsoni Macfie	2, 3, 4	
C. kelinensis Lee	4	
C. kibunensis Tokunaga	4	
C. kureksthaicus Dzhafarov	4	
C. lasaensis Lee	4	
C. liui Wirth and Hubert	3	
C. longiporus Chu and Liu	3	
C. lungchiensis Chen and Tsai	2	
C. macfiei Causey	3	
C. maculatus Shiraki	2, 4, 6	
C. majorinus Chu	1	
C. malayae Macfie	2	
C. mamaensis Lee	4	
C. marginalis Chu and Liu	3	
C. matsuzawai Tokunaga	2, 6	
C. menglaensis Chu and Liu	3	

Table 1. *Culicoides* species reported as being present in China.

Species	Reference	Host associations
C. miharai Kinoshita	2	human
C mihensis Arnaud (= *morisitai* Tokunaga)	5	
C. morisitai Tokunaga	2	
C. motoensis Lee	4	
C. nagarzensis Lee	4	
C. nipponensis Tokunaga	2, 3, 4, 5	cattle, buffalo, sheep, goat, ass, pig
C. obsoletus (Meigen)	1, 4	cattle, sheep, goats, man
C. okumensis Arnaud (= *actoni* Smith)	3	
C. orientalis Macfie	4	
C. palpifer Das Gupta and Ghosh	2, 3, 4	
C. paraflavescens Wirth and Hubert	2, 3	
C. peregrinus Kieffer	2, 3	cattle, buffalo, horse
C. pseudosalinarius Chu	2	
C. pulicaris Linnaeus	4	cattle, sheep, horse, human
C. punctatus Meigen	2	
C. qabdoensis Lee	4	
C. saevus Kieffer	3	
C. schultzei (Enderlein)/*oxystoma* Kieffer	2, 3, 5	buffalo, cattle, pig sheep goat, ass
C. sigaensis Tokunaga (= *maculatus* Shiraki)	3	
C. similis Carter, Ingram and Macfie	2	
C. sinanoensis Tokunaga	9	
C. singkianensis	7	
C. spinulosus Chu	1	
C. subfascipennis Kieffer	4	
C. sumatrae Macfie	2	
C. suzukii Kitaoka	8	
C. tbilisicus Dzhafarov	4	
C. tentorius Austen	4	
C. tianmushenensis Chu	2	
C. tibetensis Chu	1, 4	
C. verbosus Tokunaga	2	
C. yunanensis Chu and Liu	3	
C. vexans Staeger	4	

References

1. Chu Feng-I 1977
2. Chu Feng-I 1981
3. Chu Feng-I and Liu Shu-chung 1978
4. Lee Tie-sheng 1979
5. Su Genyuan and Zhou Weihan 1992
6. Zhou Weihan et al. 1991
7. Qin Qiying et al. these Proceedings
8. Li Huachun & Miller unpublished data
9. Dyce these Proceedings

Collections directly into preservatives, such as 70% ethanol, will provide specimens in good condition for taxonomic study. If collections are made into saline, any blood-engorged specimens taken can be tested to determine the source of the blood meal (Walker and Boreham 1976).

However, there are also disadvantages with this method. Light traps do not necessarily attract all species equally: indeed, even within a single species there may be differences in attraction depending on physiological state (eg. male or female, gravid or nulliparous). Light traps are not effective in cooler climates or seasons where evening and night temperatures are such that activity by *Culicoides* has ceased by the time it is dark enough for the light in the trap to be attractive.