Table 2. Synonyms for *Culicoides* species in China

Previous name	New name	Synonymised by
C. amamiensis Tokunaga	*C. sumatrae* Macfie	Wirth and Hubert 1989
C. mihensis Arnaud	*C. morisitai*	Chu Feng-I 1981
C. okumensis Arnaud	*C. actoni* Smith	Wirth and Hubert 1989
C. sigaensis Tokunaga	*C. maculatus* Shiraki	Wirth and Hubert 1989

Truck traps also have advantages. As there is no attractant involved, truck traps give an accurate indication of the flight activity patterns of species in relation to time of day, temperature, humidity and wind speed. This method is very useful for detecting daylight and crepuscular activity when cooler temperatures after dark will restrict flight and the usefulness of light traps. However, again there are disadvantages. Truck traps require a vehicle and an experienced operator, and the collection sites must be smooth enough to operate the vehicle comfortably and safely.

Table 3. Three common *Culicoides* species from the Oriental region that are confirmed or potential bluetongue vectors, with synonyms and species with which they have been confused (after Standfast et al. 1992).

C. actoni	*C. orientalis**
	*C. pungens**
	C. imperceptus
	C. okumensis
	C. robertsi
C. brevitarsis	*C. orientalis**
	C. radicitus
	C. robertsi
	C. superfulvus
*C. oxystoma***	*C. schultzei**
	C. alatus
	C. housei
	C. kiefferi
	C. mesopotamiensis
	C. pattoni
	C. punctigerus

* species with which confusion has occurred in the literature.
** synonyms for *C. oxystoma* are still in a state of flux.

For either of these two methods, it may be necessary to use a screen of mosquito netting, either around light traps or in the mouth of the truck trap. Although biting midges will pass through this screen, it will keep out the large insects that damage collections and make sorting difficult.

Animal bait

This is the most direct method of determining which species feed on livestock, and when they do so. The bait animal must be very quiet and tame enough to allow collecting from head, belly and legs.

Larval surveys

Several *Culicoides* species that are confirmed or suspected vectors breed in discrete bovine dung pats (e.g. *C. brevitarsis, C. wadai*); in dung and soil mixtures (e.g. *C. imicola*); or at the margins of dung-fouled water (e.g. *C. variipennis*). Sampling of these habitats will almost certainly produce similar connections in China.

Larval surveys allow evidence to accumulate on species that have a close connection with livestock. However, sampling may be labour intensive. If dung is to be held to allow emergence, storage space for containers of dung is required for at least two to three weeks.

Laboratory Investigations

After key species have been identified by field investigations, two approaches may be used to demonstrate a connection with BLU (or any other viruses). The first is to collect *Culicoides* in the field, sort and identify them and process pools for virus isolation. Collections can either be made 'dry', kept alive and sorted on an entomological chill table; or made into suitable saline fluid in which insects can be sorted and prepared for processing within a short time after collection (Walker and Boreham 1976). In future, the polymerase chain reaction (PCR) test will allow specimens from ethanol to be examined for the presence of viruses.

One useful technique when sorting *Culicoides* for virus isolation attempts is to select only parous specimens. (Testing nulliparous specimens is of no value, as they have not been exposed to virus infection through a blood meal.) Parous *Culicoides* can be detected by the presence of a purple pigment in the walls of the abdomen (Dyce 1969).

However, the collection of insects in the field and their processing for virus isolation is not always an efficient use of scarce resources. In the CSIRO program in the Northern Territory over 18 months during 1974/75, 170 000 *Culicoides* were collected and sorted to species for a single isolation of BLU virus (Standfast et al. 1992). The processing of field collected material gives information on vector status only if BLU virus is isolated. Also, in this type of program other viruses will be isolated too, and if there is no BLU filtering procedure in the laboratory, they will require time and resources for their identification.

The second approach is to use live insects of species suggested as potential BLU vectors and expose them directly to the virus in the laboratory. This can be done either by feeding them on an animal experimentally infected with the virus (Muller 1985) or by artificial feeding through a membrane (Davis et al. 1983). After a suitable incubation period, the insects that feed can be tested to derive an infection rate.

The next stage of the process is to determine the proportion of infected insects that transmit the virus by feeding. As infection rates may be as low as 1%, the most appropriate method of producing known infected insects is to use intrathoracic inoculation with the virus. These insects can be tested for transmission either by allowing them to feed on a host or by capillary tube feeding (Muller 1987). These laboratory procedures are described in more detail by Standfast et al. (1992).

It may still be useful to collect insects in the wild for virus processing, but generally only when the target virus is known to be active in livestock. New technologies, such as PCR, when carefully evaluated for their capacity to detect viruses in insect pools, may make this type of processing more feasible than it has been until now.

In summary, entomologists working on bluetongue vectors in China have an exciting time ahead. If we could make one recommendation, it would be to look carefully at the species that breed in association with dung. In South Africa, Nevill et al. (1992) rated potential orbivirus vectors using several criteria: of the seven species identified as the most likely candidates, six required dung for breeding. In Australia, both *C. brevitarsis* and *C. wadai*, probably the two most important vectors, breed in dung. Given that cattle are an excellent BLU reservoir and amplifier, it would be no surprise if a close relationship has evolved between the virus, the vertebrate and the vectors that are so closely connected to their hosts.

In a country as large as China with such diverse habitats, there will be much to do and much to learn in the study of bluetongue vectors. We look forward with interest to the findings that will be made as this work develops.

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Analysis of the Vector and Overwintering Hosts of Bluetongue in Anhui Province, China

Zhou Weihan*

Abstract

Twelve species of *Culicoides* are found in China's Anhui Province: *Culicoides actoni, C. arakawae, C. homotomus, C. maculatus, C. matsuzawae, C. mihensis, C. nipponensis, C. oxystoma, C. pulcaris, C. sigaensis* and two species needing further taxonomic comparison. *Culicoides homotomus* may be the vector of bluetongue virus in Anhui Province. First, *C. homotomus* is the dominant species in southern and northern epidemic areas of Anhui. Second, it prefers domestic ruminants. Third, bluetongue disease broke out in the latter part of the peak period of infection, although the peak occurred at a different time in the southern and northern epidemic areas. Both *C. homotomus* and the American *C. variipennis* belong to the subgenus *Monoculicoides*. Comparing the results of agar gel immunodiffusion (AGID) tests, particularly the positive rates of different ruminants in epidemic and non-epidemic areas, and the high positive rate in an area without sheep, it appears that local cattle and buffalo are the main overwintering hosts of bluetongue virus in Anhui Province.

AFTER the isolation and identification of bluetongue virus (BLU) in China's Anhui Province, a general survey of possible vectors and overwintering hosts was carried out in the two bluetongue epidemic areas. This survey aimed to define the dominant species of vector insects and overwintering hosts to formulate appropriate prevention strategies for bluetongue in the Province.

Materials and Methods

Culicoides midges were collected in 33 counties of Anhui Province, giving a survey rate of 37.1%. Net capture was most frequently used, as well as any other appropriate methods. Blood samples from 15 358 ruminants throughout the Province were tested using agar gel immunodiffusion (AGID) performed according to standard procedures.

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Results and Discussion

A total of 22 096 *Culicoides* were captured from 33 counties. Species numbers and distribution (Table 1) suggested that the most probable BLU vectors in Anhui were *Culicoides actoni, C. nipponensis* and *C. homotomus*, for the following reasons.

Six species were eliminated from consideration as vectors as they provided just 17 individuals. Although *C. mihensis* was found in the northern epidemic area, its activity peak occurred outside the time of the bluetongue epidemics so that species too was ignored. *Culicoides actoni* has been identified as a BLU vector in Australia, but its numbers in Anhui Province were too low to suggest a similar role without further local confirmation. The very prevalent *C. arakawae* prefers chicken blood, so it too was not considered as a bluetongue vector. There is some debate about the vector role of *C. oxystoma*, the most numerous and widely distributed species of *Culicoides* in the Province. Certainly BLU has already been isolated from this species abroad. However, the vector role for this species in Anhui Province remains unconfirmed because of differences between its activity peak and periods of bluetongue outbreaks. (A.L. Dyce has reported *C. oxystoma* as an 'outlier'.)

The most probable vector in Anhui Province was thus *C. homotomus* for the following reasons.

- *Culicoides homotomus* was the dominant species in both number and distribution, and was captured in both epidemic areas.
- It was captured around the bodies of animals and their housing, and the results of electrophoretic analyses of blood meals proved that the blood was from ruminants.
- The activity peak of *C. homotomus* fits the epidemic period of bluetongue. The latent period of both insect and virus suggested a relationship between them.

Research on *C. homotomus* as a BLU vector has been done only in Japan, because the species is confined to Japan and China. However, further study is necessary to confirm its role in China.

Even if a vector were identified, another question, that of the overwintering host, remains. Bluetongue viraemia in an infected animal lasts only 63 days, while the longest activity period of *Culicoides* was 211 days. There must be a host for this gap. A serological survey for bluetongue antibodies using AGID was carried out in Anhui (Table 2). Positive rates among ruminants were ranked, in decreasing order, as Yellow cattle, sheep, buffalo, goats and dairy cows (Table 3). The positive rates among the first three was significantly higher ($p<0.01$) than those of goats and dairy cows, but there was no significant difference ($p>0.059$) among the other three. The positive rates among cattle and buffalo were very high in some areas where there were no sheep. Combined with the long period of viraemia (reported to last 42 months) in cows, it appears that it is cattle and buffalo that are the likely overwintering hosts for bluetongue virus. Additional evidence for this conclusion comes from the significantly higher prevalence of antibodies in cattle, sheep and buffalo in the epidemic areas.

Table 2. AGID bluetongue group antibody positive rates among ruminants in Anhui Province, China.

Ruminant	No. positive/No. tested	(%)
Yellow cattle	1231/4554	(27%)
Sheep	1209/4724	(25.6%)
Buffalo	361/1464	(24.7%)
Goat	514/3811	(13.5%)
Dairy cow	37/805	(4.6%)

Table 3. Comparison of AGID positive rates in the bluetongue epidemic areas and non-epidemic areas.

Ruminants	No. positive / No. tested		Significance (P)
	Epidemic area	Non-epidemic area	
Yellow cattle	716/1446	515/2108	< 0.01
Sheep	914/2862	295/1862	< 0.01
Buffalo	282/2092	132/1782	< 0.01
Goats	3/12	3358/1452	
Dairy cows	1/1	36/804	
Total	2016/7350	1336/8008	< 0.01

Table 1. Distribution of species of *Culicoides* in Anhui Province, China.

Culicoides species	No. of insects captured (%)	No. of counties from which insects collected	Remarks
C. oxystoma	10719 (48.5)	32	
C. homotomus	6587 (29.8)	31	in both epidemic areas
C. nipponensis	3153 (14.3)	26	in both epidemic areas
C. arakawae	1423 (6.4)	25	chicken feeder
C. mihensis	120 (0.5)	7	not in southern epidemic area
C. actoni	77 (0.3)	10	in both epidemic areas
C. maculatus	17 (0.1)	–	these six species seldom seen
C. matsuzawae			
C. pulcaris			
C. sigaensis			
undefined			
undefined			
Total	22096	32	

A Comparison of the Geographic Distribution and Dynamics of *Culicoides* in Anhui Province, China

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Abstract

An investigation was carried out to help search for arbovirus vectors in Anhui Province. By geographical classification, Anhui province is an intermediate area between the Oriental and Palaearctic regions. The Changziang and Huaibe rivers run from west to east and divide the province into three natural zones; the Jianghui zone (between the Huaibe and Changjiang rivers), the Huaibe zone (north of the Huaibe River) and the Jiangnan zone (south of the Jaingsun river). *Culicoides oxystoma* and *C. homotomus* are the dominant *Culicoides* species in the whole province. Another dominant species, *C. nippenensis*, was more abundant in the Jainghuai Zone than in the Huaibe and Jiangnan zones. In all three zones, *C. actoni* comprised less than 1% of the *Culicoides* population. One species, *C. mihensis*, was collected only in the Huaibe Zone, not in the other two. The first successful collection date for *Culicoides* at the southern point (32°15'N, 18°18'E) occurs earlier than at the northern point (34°4'N, 117°2'E), and the final collection date of the season occurs later. The appearance, duration and termination of all species was respectively earlier, longer and later at the southern point, perhaps because of the 2° difference in latitude.

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A Study on Laboratory Rearing of Dominant *Culicoides* in Anhui Province, China

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Abstract

An investigation was carried out into the blood-feeding habits and breeding sites of four species of *Culicoides* (*C. schultzei, C. homotomus, C. nipponensis* and *C. arakawae*), which are suspected vectors of infectious diseases in Anhui Province. Techniques for rearing *Culicoides* in the laboratory were evolved, with a success rate of 4.7% with *C. nipponensis* adults.

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A Comparison of Methods for Isolating Arboviruses from Insects

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Abstract

Arbovirus isolations were made from 97 field samples from certain *Culicoides* species. Various methods were used, including inoculation and propagation in yolk sacs of embryonated eggs, in suckling mice, and on cells either of a single kind or of two kinds in various ways. The rates of success for virus isolation were 8.9% in embryonated eggs; 21.6% in mouse brains; 28.9% in cells of a single kind; and, most effective, 62.5% in cells in alternate ways.

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Application of the Polymerase Chain Reaction (PCR) Test with Insects in Studying Bluetongue Virus Activity

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Abstract

The polymerase chain reaction (PCR) test for bluetongue viruses offers a sensitive and specific means of detecting the presence of bluetongue nucleic acid in samples. Two different tests have been developed so far. In the first, a genome fragment from the bluetongue viral RNA3 segment is amplified: RNA3 codes for the capsid protein VP3, and is substantially conserved among bluetongue serotypes. Within the limits of test sensitivity, a single test can determine the presence or absence of a bluetongue virus. In the second type of test, PCRs for each of the BLU serotypes have been developed, based on serotype-specific domains in RNA2 segment coding for VP2, the outer coat protein of the virus. Either test can be applied to insect specimens, but first the test must be standardised so that its sensitivity in detecting one infected insect in insect pools of various sizes is known. In monitoring programs, PCR offers the opportunity to detect bluetongue virus in insects collected from remote locations where sentinel cattle cannot be used for routine sampling. In research, studies of insect vector–virus–mammalian host cycles of infection can be attempted, because PCR gives an efficient way of detecting the viruses in the insect phases of their life cycles. Both PCR applications are currently being developed in the Northern Territory of Australia.

ARBOVIRUSES, by definition, are spread among mammalian hosts by insect vectors, with the virus undergoing an obligatory cycle of replication in the insect as well as in the mammalian host. Epidemiological studies of arboviruses must include the insect phase of the infection cycle before a full understanding is possible. However, including insect studies adds substantially to the required resources: a qualified entomologist with specialist knowledge of *Culicoides* species must be available at the right time and place, as must the equipment for handling insects. If live or cold-preserved insects are needed, for virus isolation or vector studies, such equipment needs can be limiting.

Polymerase chain reaction (PCR) amplification of specific sequences of nucleic acid (Saiki et al. 1988) can be applied to alcohol-fixed specimens, and thus offers new opportunities and flexibility to studies of associations of infectious agents with insects. Insects can be collected by light trap into 70% alcohol at near or remote locations with minimum facilities, be stored cheaply and without the need for specialised equipment, and transported to an entomologist for sorting and identification at a mutually convenient time (Daniels and Sendow these Proceedings).

A prerequisite for studies with any particular virus is a PCR test of known and appropriate specificity. Considerable developmental work has been reported on such PCR tests for bluetongue viruses (BLU) in the Australian region (Gould et al. 1989; Gould and Pritchard 1991; McColl and Gould 1991, 1994; McColl et al. 1994; Pritchard et al. 1995). In summary, a PCR test that detects nucleic acid from all known bluetongue viruses has been developed, based on amplification of nucleic acid from the virus RNA segment 3 which codes for the well conserved inner capsid protein VP3. Sensitivity of this primary PCR has been increased by the addition of a sequential round of amplification using 'nested' primers: these

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initiate a PCR for a smaller fragment of the gene contained within the segment amplified in the first reaction, giving a nested PCR. In addition, primary PCRs have been developed for specific Australian BLU serotypes, based on the serotype-specific VP2.

Importantly, nucleotide sequences in BLU RNA segments have been shown to vary between isolates, even of the same serotype. Relationships can be detected among isolates: those with closer sequence homology tend to be derived from one geographic area. These relationships are independent of serotype. After information on a sufficient number of isolates of known origin and date of isolation has been analysed it is possible to nominate the probable geographic origin of new isolates based on their sequence relationships to those already known. These relatively new observations have led to the concept of 'topotyping' (Trent et al. 1981; Gould et al. 1989; Pritchard et al. 1995).

The present studies in the Northern Territory, promoted by the Exotic Animal Disease Preparedness Consultative Council (EXANDIS), are part of a collaborative project in technology transfer between the Berrimah Agricultural Research Centre and the CSIRO Australian Animal Health Laboratory, Geelong, Victoria. Our purpose is to devise strategies for the application of newly available molecular techniques to epidemiological studies of bluetongue viruses in Northern Australia, as well as more efficient monitoring strategies. In this paper we describe approaches being developed to apply PCR technology to the detection of bluetongue viruses in insects.

Potential Applications for PCR Investigations of Insects

In large countries such as Indonesia and Australia, much is already known of the BLU fauna from isolations of viruses from sentinel animals (Sukarsih et al. 1993, Melville et al. these Proceedings; Sendow et al. these Proceedings). Light trap collections of insects may offer a preferable monitoring strategy for specific purposes under some circumstances for example, to confirm the presence of virus in an area where monitoring of sentinel animals is not practical for logistical or social reasons. In this case, insects need not necessarily be identified to species, nor should having a blood meal lead to exclusion, since the aim is not to make inferences about vectors but simply to confirm the presence of virus at the sampling location. Efficiency of virus detection would increase with the sorting of insects into pools of potential vectors.

Sequence analysis of the PCR product, or topotyping, could give additional information as to whether the detected virus was potentially a recent introduction to the sampling area, as long as the expected topotype was known from previous experience (McColl et al. 1994). Detection of a topotype of concern would lead to further monitoring to attempt to describe its distribution.

If insect collections are reliably sorted to species, and according to normal protocols for virus isolation before PCR analysis, one can establish specific associations of viruses with wild caught insect species, one of the steps in determining vector status.

These PCR applications should also be useful in epidemiological studies of the dynamics of natural viral infection cycles in vector–mammalian host ecosystems. Such studies are not presently attempted because they require excessive amounts of limited resources. At the Coastal Plains Research Station in the Northern Territory, each week insects are collected and viruses isolated from sentinel cattle, with great efficiency (Gard and Melville 1992; Melville et al. these Proceedings). In this setting, it is proposed to plot the temporal relationship between infections in the vector species present and the flux of viraemia in the sentinel cattle group. Strategies will also be investigated to determine the infection rates in various vector species as the wave of virus infection moves through the sentinel herd. Such data will provide an understanding of events that may allow predictions of the probabilities of spread of any BLU serotype, given the prevailing population characteristics of the vectors present at the time.

Limitations to the Application of PCR in Insects

Sensitivity of PCR in insect homogenates

As with any PCR test, proper application depends on a knowledge of its sensitivity in detecting virus. With insect material, PCR may be less sensitive than with other specimens. Three major concerns must be identified. First, with large pool sizes dilution factors may become important. Second, insect homogenates may contain inhibitors of the various PCR processes (Sellner et al. 1993): the probability of high levels of RNAses interfering with RNA extraction has been noted (Noriega and Wells 1993). Third, sensitive procedures must be developed and their sensitivity known before serious application is attempted, otherwise negative results will be difficult to interpret. Knowledge of the probability of detecting one infected insect in pools of various sizes is of particular importance.

The biology of the virus in the insect

As with the isolation of viruses from insects, the detection of virus by PCR does not confirm vector

status. To do the latter, vector competency studies must confirm that the species is able to transmit the virus biologically. Sources of viral RNA that are theoretically detectable by PCR include possible survival or retention of virus after a blood meal in the insect gut, or possible multiplication of the virus without concomitant ability to transmit. Muller (pers. comm.) has recommended studies of the kinetics of PCR-detectable material in experimentally infected insects after a blood meal to develop an appreciation of likely PCR observations during different phases of the insect's life cycle.

Another possible biological constraint to applying PCR in studies of virus infections in *Culicoides* populations is the low infection rate observed with some species. For example, although *C. fulvus* had infection rates of 43% for BLU1 and 64% for BLU20, *C. brevitarsis* had infection rates of just 0.2% to 0.3% for BLU1, 20 and 21 (Standfast et al. 1985). Detecting virus-infected insects under such circumstances would necessitate the processing of either pools of extremely large sizes (which that may be confounded by factors mentioned above) or a large number of smaller pools, reducing the cost-effectiveness of the technique. However, PCR studies on natural populations may give an indication of infection rates in nature.

Materials and Methods

To assess PCR sensitivity in the detection of infected insects, we inoculated insects with virus and added them to uninoculated insects in varying pool sizes. Insects were caught in an updraught light trap at Coastal Plains Research Station (12° 39'S, 131°E,). *Culicoides peregrinus* was chosen as the test species because of its large size (making inoculation easier), its abundance and its reported ability to support BLU replication (Standfast et al. 1985). Individual *C. peregrinus* were sorted from the collection and held in an incubator for 24 hours at 26° C and 85% relative humidity, with access to 10% sucrose solution as a food source. After 24 hours, surviving insects were inoculated (Muller 1987) with BLU1 (CSIRO 156 strain) at a titre of 6.75 $\log_{10}$ $TCID_{50}$. After inoculation, insects were returned to the incubator and held for varying times post-inoculation depending on survival. The longest surviving insect was held for 20 days post-inoculation.

Pools were made of inoculated and uninoculated insects, at ratios varying from 1:5 to 5:50. These insect pools were held either at –20°C or at 4°C until processed. Insect pools were ground in 50 mL of sterile distilled water using a disposable mortar and pestle for each sample. The sample was made up to 50 mL, digested RNA extracted, cDNA prepared, primary and nested PCRs performed and products analysed (McColl et al. 1994). Primers used were based on RNA3 of BLU1 (Pritchard et al. 1994).

A similar PCR technique was applied to wild-caught alcohol-fixed insects. Insects were caught in a downdraught light trap into alcohol. Traps were set weekly at Coastal Plains Research Station and samples were examined from October to April. The PCR results were compared with virus isolations from cattle over the same period.

Results and Discussion

Although there was a slight tendency for PCR reactions to occur in the smaller pools of experimentally infected insects, no clear trend has emerged (Table 1). Further studies are necessary, replicated to control for possible failure of viruses to propagate in inoculated insects.

Table 1. PCR detection of bluetongue-inoculated *Culicoides* in pools of uninfected *Culicoides*.

Ratio of infected : uninfected	No. of pools tested	No. of pools positive by PCR
1 : 5	4	4
2 : 5	3	1
1 : 10	2	1
2 : 10	3	1
5 : 10	1	1
1 : 20	2	1
2 : 20	2	1
1 : 50	1	0
2 : 50	1	0
5 : 50	2	2

In the pilot investigation of field-caught insects (Table 2), BLU was detected in all four species of known vectors (*C. actoni, C. brevitarsis, C. fulvus* and *C. wadai*), as well as in separate pools of *C. peregrinus* and *C. oxystoma* (data not presented). The findings represent the first PCR detection of BLU in field-caught insect specimens for all these species except *C. wadai*, which has yielded PCR product previously (McColl et al. 1994). The result for *C. actoni* was the first demonstration of BLU in wild-caught insects for that species, with the other species having been implicated previously through various isolation techniques (Standfast et al. 1985) or, in the case of *C. wadai*, by PCR.

Quite small pools of *C. wadai* and *C. fulvus* were PCR positive, indicating quite high infection rates at that time. BLU was detected by PCR in pools of 25

and 50 *C. brevitarsis*, even though only a few pools were processed. This suggests the technique could be applied to that species even with its predicted lower infection rates.

Table 2. Summary of *Culicoides* species showing positive for bluetongue virus by PCR.*

Culicoides species	Month	No. of insects	No. of pools	No. of pools positive by PCR
C. brevitarsis	October	160	5	0
	November	160	5	0
	December	370	11	3
	January	100	2	0
	February	50	1	1
C. wadai	October	5	1	0
	November	2	1	1
	December	40	4	2
	January	91	4	2
	February	25	1	0
C. fulvus	October	1	1	0
	November	1	1	0
	December	3	2	1
	January	102	4	4
	February	50	1	0
C. actoni	December	12	1	1
	January	54	2	1
	February	4	1	0

*Preliminary data

The results can be further assessed to show how studies of the dynamics of virus infections in a natural vector–mammalian host ecosystem might be conducted. Observations were made during the time that BLU21 was known to be circulating among a group of sentinel steers at Coastal Plains Research Station. The first isolation, from a single infected animal, was on 20 October 1994, and the last isolation, when again only one animal of the group was infected, was on 9 March 1995. Periods of viraemia in infected steers lasted from one to four weeks, as assessed by virus isolation. All 20 steers were infected during this infection cycle (Fig. 1).

A means of identifying the peak of the infection cycle in the steers was needed, to indicate the pool of animals infected each month resulting from insect infections, and in turn contributing to the number of insects infected. The number of viraemic animals each week was determined, and monthly totals calculated, giving values for 'viraemia-weeks' per month. This measure (Fig. 1), analogous to 'person-days' per month (eg. working on a project), was plotted to give a clear representation of the peak of the infection

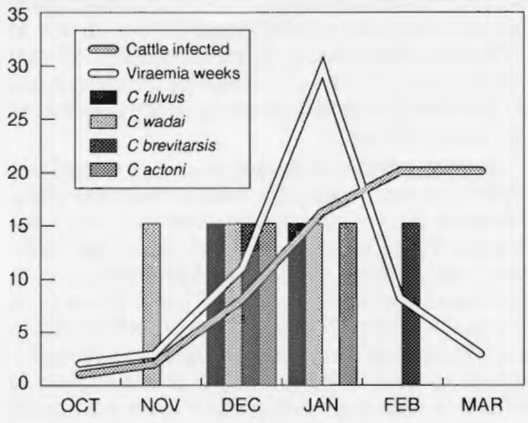

Figure 1. Dynamics of BLU 21 infection in cattle and insects.

cycle, compared with the cumulative total of the number of animals infected.

Also plotted were the monthly data on the vector species identified as infected by PCR (Fig. 1). When the infection cycle began, only *C. wadai* was identified as PCR positive. As the cycle gained momentum, all four vector species were implicated. As long as adequate numbers of insects of each species are tested each month, such data may show which vector species is involved in introducing the infections to the sentinel group. Similarly, subject to certain assumptions, the vectors involved in propagating the wave of each infection may be determined. On this occasion there was a maximum (100%) incidence of infection of the mammalian host sentinels, but this is not always, or even usually, the case (Daniels and Sendow, these Proceedings). The comparative involvement of vectors during less efficient cycles of infection could be observed in future studies. Also, as certain BLU serotypes and two of the four vectors are restricted to the north of the Northern Territory, it will be important to establish whether all four vectors are equally involved in the propagation of all eight Australian serotypes, or whether apparent vector–serotype preferences can be distinguished (Daniels and Sendow, these Proceedings).

These preliminary studies have provided novel observations of aspects of BLU epidemiology in the Northern Territory. There seems adequate justification for developing these approaches further, by defining the sensitivity of PCR more precisely and by

developing epidemiologically-sound sampling protocols on the basis of that information.

Acknowledgments

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Entomology Workshop

Convened by H.A. Standfast and M.J. Muller

Collection Techniques

Participants discussed the use, and application to various species of insect, of light traps, truck traps, manitoba traps and mechanical aspirators, with respect to the collection of *Culicoides* for manipulation in the laboratory. One important aspect was the requirement to minimise stress on the insects during the collection process so they would survive the 10 to 15 days required for laboratory experiments.

Infecting *Culicoides* in the Laboratory

Techniques for infecting insects were discussed, including methods for feeding insects on viraemic animals (and restraining the latter); techniques for feeding *Culicoides* blood virus mixtures through a membrane; and techniques for inoculating *Culicoides* with virus suspensions.

Laboratory Transmission

Systems similar to those used for feeding *Culicoides* on viraemic sheep were outlined. M.J. Muller described a technique he had developed for collecting saliva from infected *Culicoides* and for recovering virus from the saliva. Bluetongue viruses can be identified by direct cultivation or polymerase chain reaction (PCR).

Vector Efficiency

Techniques for measuring vector competence were outlined. The traditional method, of feeding insects on infected sheep and transmitting the virus to susceptible sheep, requires insect-proof animal accommodation. A second method, which can be used in a normal laboratory, involves feeding insects on blood virus mixtures through a membrane, then inducing the infected insects to feed on serum saline in a capillary. The saliva-contaminated serum saline is then assayed for the presence of virus either by inoculating tissue culture or by the use of PCR.

Control of *Culicoides*

For too long control agencies have concentrated on the use of vaccines, ignoring the possibility of controlling the vector. In the last two decades several techniques developed to control mosquitoes have shown promise for the control of *Culicoides*. Some systemic insecticides are useful, while some vector species can be controlled simply by changing hygiene and farming practices. For nearly a quarter of a century the philosophy of integrated control has been applied to disease management. It is time it was applied to bluetongue.

Conclusion

Symposium delegates attending the Entomology Workshop participated actively in the discussions and hoped that, at the next Symposium, some results of studies on vector competence will be available for discussion. One outcome of the Workshop was the identified need for a Chinese entomologist to receive specialist training on the taxonomy and biology of *Culicoides* to advance the task of identifying which species of *Culicoides* in China are bluetongue vectors. Table 1 provides a list of *Culicoides* species fully identified from cleared and mounted species.

Table 1. Identification of *Culicoides* species collected in China between 1987 and 1991. (Collected by S.S. Davis, Li Hauchun, M.J. Muller and T.D. St. George: identified by A.L. Dyce.).

Species	Songhua[a] 16 Jun 1987	Longjiang[b] 25 May 1989	Lufengchun[c] 20 Oct 1991	Yiliang[d] 21 Oct 1991
C. (Avaritia) imicola Kieffer			+	
C. (Avaritia) jacobsoni Macfie			+	+
C. (Avaritia) maculatus (Shiraki)			+	+
C. (Avaritia) sp (?) *obsoletus* Meigen			+	
C. (Avaritia) orientalis Macfie			+	
C. (Avaritia) sp. nr *pastus* Kitaoka			+	+
C. (Avaritia) sinanoensis Tokunaga	+			
C. (Avaritia) suzukii Kitaoka				+
C. (Culicoides) pulicaris group	+		+	+
C. (Monoculicoides) homotomus Kieffer	+			
C. (Hoffmania) nipponensis Tokunaga		+		
C. (Hoffmania) sumatrae Macfie			+	
C. (Meijerehelea) arakawae (Arakawa)		+	+	
C. (Trithecoides) sp. (? *humeralis/palpifer*)		+	+	
C. (Remmia) oxystoma Kieffer		+	+	
Forcipomyia sp.				+

[a] Songhua Dairy, north of Harbin, Heilonjiang Province 45°50'N, 126°5'E.
[b] Lufengchun, Yunnan Province 25°10'N, 102°E.
[c] Yiliang, Yunnan Province 25°N, 103°20' E.
[d] Longiang Experimental Station, southwest Foshan, Guangdong Province 22°50'N, 113°10'E.
+ Species present in collections made at locations [a, b, c] and [d].

Bluetongue Vector Surveillance in Yunnan and Sichuan Provinces of China

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Abstract

Bluetongue is a virus of ruminants transmitted by *Culicoides* species. In 1994, the surveillance and studies of bluetongue vectors began in Shizong and Kunming in Yunnan Province, and in Wuxi County in Sichuan Province. Twenty-three species of *Culicoides* have been collected, of which at least six are bluetongue-related vectors. Five species (*Culicoides actoni, C. brevitarsis, C. imicola, C. maculatus* and *C. orientalis*) are found in Yunnan, while another species, *C. fulvus*, is found in Sichuan.

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Virology, Serology and Pathogenesis

Introduction

BLUETONGUE has many vertebrate hosts, including ruminants of several species, both domesticated and wild. Our understanding of the effect of bluetongue on these hosts has been clouded by the unwarranted extrapolation from data from a single source some years ago. Now there is solid evidence that bluetongue is an infection or disease of finite duration.

Bluetongue is a disease produced by a group of closely related viruses which circulate in ruminants in a vast area of the world. Humans have domesticated only a few of the species infected by these viruses. Among these domesticated species, only certain breeds of sheep experience the more severe effects on their vascular endothelium which produce the clinical effects that we call bluetongue disease. We have not yet identified the differences between hosts which mean that, when infected with the same virus, some respond with disease and some do not.

In these Proceedings, very strong evidence is advanced to suggest that bluetongue viruses persist for less than 150 days, and that this is directly determined by the life span of red cells to which virus adsorbs during a short replication period. The presence of neutralising antibodies in the plasma of infected ruminants, and the declining titre of virus, makes the real infective period when suitable *Culicoides* vector species feed on the infected animal very much shorter.

The recognition that bluetongue virus is stable in blood samples held above freezing temperature, and the improvement of cultural techniques, are leading, in China and Southeast Asia, to the accumulation of many strains of a wide range of serotypes from hosts of known history. This new wealth of material is allowing the evaluation of more recently developed serological methods, in particular the competitive enzyme linked immunosorbent assay (cELISA).

Similarly, the role for polymerase chain reaction (PCR) is beginning to come into focus. Its use can speed up screening techniques remarkably, whether applied to the blood of insects or ruminants. However, PCR does not replace methods which yield viable viruses for typing or testing for pathogenicity. Approaches using combinations of PCR, virus isolation and cELISA need encouragement throughout the Asian-Pacific region in this exciting period of the exploration of bluetongue.

The Pathogenesis of Bluetongue Virus Infection of Cattle: a Novel Mechanism of Prolonged Infection in which the Erythrocyte Functions as a Trojan Horse

N.J. MacLachlan*

Abstract

Bluetongue is an arbovirus disease of ruminants. Cattle are a reservoir host of bluetongue virus (BLU). Virus amplification occurs in infected cattle: these animals then have a prolonged infection during which they provide a source of virus for the haematophagous midge (*Culicoides*) vector. Primary replication of BLU within infected cattle occurs in the lymph node draining the site of virus inoculation. Virus is then disseminated to secondary sites such as the spleen. Initially, virus released into the circulation promiscuously associates with all blood cells, in titres proportionate to the relative concentration of each cell type in blood, but late in the course of infection virus is exclusively associated with erythrocytes. Viraemia persists for up to eight weeks as determined by virus isolation, whereas it may be detected by polymerase chain reaction (PCR) analysis for up to 140 to 160 days after infection, but not thereafter. Association of BLU with blood cells, especially erythrocytes, facilitates prolonged viraemia in infected cattle but does not affect other potential mechanisms such as antigenic drift of BLU or deficits in the host's antiviral response. BLU replicates in bovine monocytes and CD4+ T cells *in vitro* but not in other blood cell types. Significantly, BLU rapidly binds bovine erythrocytes through a highly specific interaction. Virus particles then persist for prolonged periods in membrane bound invaginations. This novel interaction of BLU and bovine erythrocytes may allow cattle to function as a reservoir host for bluetongue virus, by facilitating both the prolonged viraemia in infected cattle and infection of the haematophagous insect vector.

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Research on the Basic Properties of Chinese Bluetongue Viruses

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Abstract

Research on different local strains of bluetongue viruses (BLU) from Yunnan, Hubei, Sichuan, Inner Mongolia and Xinjiang Provinces has shown the following characteristics. Under the electron microscope, the virions present a spherical shape with no envelope and a diameter of 65–70 nm. According to polyacrylamide gel electrophoresis (PAGE), the BLU genome comprises 10 RNA segments with an electrophoretic profile of 3.3.3.1., but there are differences among strains. The physical and chemical properties of the Y33F5 strain from Yunnan, passaged through sheep, were investigated. This strain was not inactivated under conditions of 1.5% methanol, 3% Na_2CO_3, 3% lysol and chloroform, or being held at 56°C for 30 minutes. However, it was inactivated completely under conditions of 3% carbolic acid, 75% ethanol, 39% NaOH, 0.2% peracetic acid and 2% ether, or being held at 80°C for 15 minutes. Both Y33 and Y55 strains of BLU1 from Yunnan retained their virulence stability for at least 28 months after treatment with citric acid anticoagulant and storage at 4°C. After 22 passages, the viruses remain pathogenic to sheep with an ID_{50}/5 mL of 2.5 and 2.75 (log_{10}) respectively.

BLUETONGUE viruses (BLU) are classified as orbiviruses in the family Reoviridae. The virus particles measure 65–70 nm in diameter and are icosahedral in shape. Mature virus has no envelope and contains seven structural proteins. The viral nucleus comprises 10 double stranded RNA segments. Methods of BLU isolation, culture, physical and chemical properties, purification and experimental infection of animals have been studied systematically all over the world. In China, basic studies started in 1979 after the first isolation of a bluetongue virus.

The first step in genomic research is purification of the virus. This depends on the physical, chemical and biological properties of the virus. Bluetongue viruses are resistant to ether, chloroform and F113, and sensitive to low pH but stable at high pH. Standard purification procedures involve abstracting the material, partly de-proteinising and de-lipidising with a lipophilic solution, and centrifuging in discontinuous gradient sucrose or in caesium chloride many times to obtain a highly purified protein (Fernandes 1959; Els and Verwoerd 1969; Verwoerd 1969; Bowne 1971). Problems with the procedure include overelaborated operation needing time-consuming and expensive equipment; unsatisfactory results from unstable viruses (Els and Verwoerd 1969); and toxicity of caesium chloride causing losses of BLU activity (Els and Verwoerd 1969). As we wanted to establish a method without these disadvantages, we used the molecular sieve and found this satisfactory.

Blood cells were the best materials for both animal inoculation and virus purification. During the initial stages of infection, BLU is attached to the surface of red cells (Alstad et al. 1977). Most attachment occurs on the ninth day post infection, just before appearance of neutralising antibodies (Klontz et al. 1962). While infection can be achieved by intradermal inoculation (Fernandes 1959), a subcutaneous inoculation of 1 mL high titre infectious virus is the main method used in animal experiments (Bowne 1971). Resistance of BLU to storage temperatures is high: activity

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in uncoagulated blood can last 25 years at room temperature (Neitz 1948). Many other reports confirm these findings (Bowne 1971; Guo Zaijun et al. 1989; Lin Lihui et al. 1989; Li Zhihua et al. 1989; Zhang Nianzu et al. 1989a). Experiments on inoculating sheep and detecting BLU in different organs, as well as resistance to general disinfectants and temperature have been conducted by Bowne (1971), Zhang Nianzu (1989a) and Qin Qiying et al. (1991).

Our experiment aimed to measure the passaging of a Yunnan virulent BLU strain, its preservation and the 50% infection dose (ID_{50}) of sheep.

Materials and Methods

BHK21 cells were cultured and passaged by generally used methods, with the following BLU isolates:

- Y863 isolated from a sheep in Shizong farm, Yunnan
- WFX from Zhaochong farm, Hubei (Zhang Nianzu et al. 1989b)
- NMFx from Bayannur region, Inner Mongolia (Li Zhihua et al. 1989)
- SWFx from a newly opened-up region in Sichuan (Lin Lihui et al. 1989)
- SFx from a goat in Xinjiang (Guo Zaijun et al. 1989)
- BLU10 and BLU17 as positive controls imported from USA.

All the viruses caused obvious cytopathic effect (CPE) in BHK21 cell sheets. We used the method of virus amplification, harvest and concentration described by Qin Qiying et al. (1991), abstracting by adding an equal volume of F113 to concentrated virus and storing in a 4°C waterbath.

We used a Sephadax-G200 (Pharmacia, F113) molecular sieve (supplied by Shanghai Chemical Reagent Supply Company) and TES elutant. Collectors were partly used for collection with a 280 nm detecting solution for detecting nucleic acid and graphs were recorded automatically. The ID of each peak was evaluated and examined by electron microscopy and PAGE, and the virus activity tested by inoculation into BHK21 cells.

The five domestic isolates from Yunnan, Hubei, Sichuan, Inner Mongolia and Xinjiang (Li Gen and Peng Kegao 1989) were observed by electron microscopy after purification. After purification, the nucleic acid of each BLU was analysed by SDS-PAGE with the international standard isolates as controls. After treatment with general disinfectants such as 1.5% methanol, 3% Na_2CO_3, 75% ethanol, 39% NaOH, 0.2% and 2% peracetic acid and ether, or being kept at 80°C for 15 minutes, isolate Y33F5 was inoculated to sheep into determine its characteristics and resistance to treatment.

Passage, storage and activation detection

For infectivity tests we used healthy cross-breed sheep, aged 6 to 12 months, from a bluetongue-free area.

Original virus

For passage tests, we used virus isolated in July 1979 from sheep with natural bluetongue, numbers 33 (Y33) and 35 (Y35). Two groups of three sheep each were inoculated subcutaneously with 5 mL of whole blood containing Y33 (passage 18) and Y55 (passage 5) respectively. Blood was collected 5 to 18 days post-infection and stored or inoculated into experimental animals.

To examine storage resistance, 12 sheep blood samples stored at 4°C were used to confirm infectivity. Except for one sheep inoculated with original blood from Y55, 22 sheep were inoculated with 5 mL of 12 samples from different passages, using two sheep per inoculum.

To analyse activity, we used virulent viruses Y226F10 and Y33-505F5 from sheep used for passaging. Two trials were conducted. In the first trial, 16 sheep (four groups of four) were inoculated with 5 mL of inoculum in dilutions of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} (diluent was 5% glucose–saline). In the second trial, 12 sheep (four groups of three) were inoculated with 5 mL inoculum at the same dilutions as above but after three sets of washing with saline.

Criteria for sheep experiments

All experimental animal were kept in isolation, their body temperature measured twice daily, and leucocytes measured every two days. Clinical signs were recorded daily for 14 days. All experimental sheep showing an elevated body temperature, a leucocyte decline of one-third and other clinical signs were scored as strong positives (++), while those with subclinical infection were scored as positive (+). The remaining sheep, with no changes in body temperature and leucocytes, were considered negatives (–). The infectious dose at 50% endpoint was calculated by standard methods.

Results and Discussion

Most of the viruses examined were complete and spherical in shape, and unenveloped. The size of virions was uniform at 65–70 nm in diameter. There were no significant differences among isolates. The morphological appearance of bluetongue viruses could be of some diagnostic value, if seen in tissues or blood, but supplementary serological or biochemical tests are necessary to put them into their groups.

The bands of five isolates from SDS-PAGE were 3.3.3.1, which agreed with standards for bluetongue.

There were slight differences between strains but none between serotypes. SDS-PAGE was thus a reliable method to determine strain differences.

While 1.5% methanol, 3% lysol and exposure to 56°C did not kill the viruses, 75% ethanol, 3% Na_2CO_3, 3% NaOH, 0.2% and 2% peracetic acid and ether, or a temperature of 80°C for 15 minutes, effectively inactivated them (Table 1).

Table 1. Chemical resistance and physico-chemical properties of Chinese bluetongue strains.

Treatment of virus	No. of inoculated sheep	No. of sheep with blue-tongue	Incidence rate	Effective rate of treatment
3% NaOH	2	0	0/2	2/2
75% ethanol	4	0	0/4	4/4
2% peracetic acid	4	0	0/4	4/4
0.2% peracetic acid	4	0	0/4	4/4
3% carbolic acid	2	0	0/2	2/2
ether	4	0	0/4	4/4
3% lysol	2	1	1/2	1/2
3% Na_2CO_3	2	1	1/2	1/2
chloroform	2	1	1/2	1/2
56°C for 30 min	2	1	1/2	1/2
80°C for 15 min	4	0	0/4	4/4
1.5% methanol	2	2	2/2	0/2
Control (whole blood)	2	2	2/2	
Control (saline)	4	4	4/4	

Bluetongue strains from different passage levels induced typical clinical symptoms of bluetongue. The mortality of sheep caused by high passage virus was less in comparison to low passage virus. Strain Y33 is an obvious example. The appearance of other clinical signs such as swollen head and ears, erosion in nostrils, oedema and haemorrhage of the tongue and mouth mucosa declined as the passage level increased.

The duration of storage at 4°C was from 6 to 28 months. There was little difference between each passage in virulence value (Table 2). The virulence of isolates titrated in sheep gave an ID_{50} for Y226F10 of $10^{2.25}$/5 mL and for Y-505F5 was $10^{2.75}$/5 mL (Table 3).

Table 2. Storage durability of Yunnan strains of bluetongue virus.

Strain and passage	Storage time (months)	Sheep inoculated	Clinical diagnosis (+/–)
Y-F247, 47, 57	28	2	++ ++
Y-F3-64, 46	26	2	++ ++
Y-F8-125, 315	18	2	+ ++
Y-F15-339, 348	18	2	++
Y-F16-459	10	2	++ ++
Y-F10-293	6	2	++
Y55-F3-457, 458	6	2	+ ++
Y55-F4-472, 479	10	2	++ ++
Y55-F2-207	9	2	+ +
Y55-F2-447	8	2	++
Y55 experimental inoculum	6	2	++ +
Y55 experimental inoculum	18	1	++

Table 3. Infectivity of Yunnan virulent strain of bluetongue virus.

	Dilution	Inoculated animal
Y226F10	10^{-1}	4/4
	10^{-2}	4/4
	10^{-3}	1/4
	10^{-4}	0/4
	ID_{50}	$10^{2.5}$/5 mL (2 January 1981)
Y505FS	10^{-1}	3/3
	10^{-2}	3/3
	10^{-3}	1/3
	10^{-4}	0/3
	ID_{50}	$10^{2.75}$/5 mL (26 June 1982)

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Identification of Local Chinese Strains of Bluetongue Virus to Serotype

Zhang Nianzu, Peng Kegao, Li Zhihua, Zhang Khaili, Li Huachun, Hu Yuling, Li Gen, Zhang Fuqiang, Li Xinrong and Ben Jin*

Abstract

Since the first outbreak of bluetongue disease in Yunnan in 1979, bluetongue viruses (BLU) have been isolated from sheep in Yunnan, Hubei, Sichuan, Anhui, Shandong and Shanxi Provinces, from goats in Xinjiang and Inner Mongolia, and from cattle in Gansu. These virus strains were identified to group by agar gel immunodiffusion (AGID), immunofluorescence (IF) and agar gel electrophoresis. Eight of the Chinese strains were identified to serotype by microneutralisation tests. These showed that strains Sx1 from Yuncheng County in Shanxi, Sx2 from Jiansu County in Shanxi, Y863P 12 from Yunnan, X27 from Xinjiang and Yc from *Culicoides* trapped in the animal laboratory in Kunming were serotype BLU1. Both WP7 from Hubei and SWP7 from Sichuan Province were BLU16. Early results indicate that NMP11 from Inner Mongolia might be BLU17 but further confirmatory tests are required.

BLUETONGUE epidemic sites were found in four provinces of China and AGID-positive animals in 29 provinces. Bluetongue virus (BLU) was isolated after the first outbreak of disease in Yunnan Province (Zhang Nianzu et al. 1993). Initial reports identified at least four serotypes existing in China using the tests (AGID, immunofluorescence and RNA-PAGE) available at that time (Zhang Nianzu et al. 1991). This paper describes the identification of local isolates from different provinces of China by microneutralisation tests.

Materials and Methods

Isolates

Eight isolates and specimens were tested:

- Y863P 12 from Yunnan (isolated from a sheep in 1979)
- WP7 from Hubei (isolated from a sheep in 1988)
- Sx 2 from Shanxi (isolated from a sheep in 1994)
- Sx 1 from Shanxi (BHK21 cell culture of blood collected from a sick sheep in 1993)
- SWP 9 from Sichuan (isolated from a sheep in 1988)
- X 27 from Xinjiang (isolated from a goat in 1989)
- NMP 11 from Inner Mongolia (isolated from a goat in 1989)
- Yc from Yunnan (isolated from *Culicoides* in 1993).

Antisera

Four sets of antisera were used:

- Standard sera of BLU1-24 (from Veterinary Research Institute, Onderstepoort, South Africa, provided by the China–Australia Bluetongue Project), reconstituted with distilled water then stored at –20°C.
- Negative control, prepared from newborn calf serum.
- Sx 1 positive control sera, prepared from the blood of Shanxi bluetongue-diseased sheep inoculated into experimental sheep in this laboratory.
- Standard positive controls, BLU1, 15, 20 and 23 provided by Berrimah Agricultural Research Centre, Australia.

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Procedure

1. Microneutralisation: according to the standard protocol of Gard and Kirkland (1993).
2. Primary screening of serum: standard positive sera diluted with PBS at 1:20, then sera tested, and any positive sera selected for titration to identify serotype.
3. Outline of identification procedure: positive sera serially diluted at 1:1; Sx 1 serum diluted at 1:5 as the initial concentration, then serially diluted at 1:1; reaction volume of 25 µL; control plus 25 µL; tested serum plus 100 µL cell (concentration at 2.5×10^{-5}/mL); reading control as 100 $TCID_{50}$. Plates read daily, and wells with any cytopathic effect (CPE) recorded as positive, those without CPE as negative.

Results

The results for most of the isolates were interpreted easily (Table 1; positive results only). Isolates Y863, Sx1, Sx2, Yc. and X27 were clearly BLU1 as they reacted strongly to the BLU1 standard control. Isolates WP7 and SWP 9 were strongly neutralised by standard BLU16 and BLU3. There was a very slight cross-reaction with BLU13 but no neutralisation with BLU1–12, BLU14–15 and BLU17–24. However, when titrated there was a four-fold greater titre with the antiserum to BLU16. Isolate NMP11 was strongly neutralised by BLU17 antiserum, partially cross-neutralised by BLU20 and 22 antisera and very slightly inhibited by BLU4 and 11.

Discussion

Our results support the conclusions of primary screening and serum surveys, that is, that at least two separate serotypes are circulating in China. Five isolates from Shanxi, Yunnan and Xinjiang are BLU1. Isolates SWP9 (Sichuan) and WP7 (Hubei) are BLU16 (significant neutralisation with BLU16 standard control, and lower partial cross reaction with BLU13). The serotype of isolate NMP11 needs further confirmation, as there are contradictory results from primary screening (strong reaction with BLU17) and from microneutralisation survey tests (no BLU17 antibodies detected in the area). Thus NMP11 is only provisionally identified as BLU17.

All results basically confirmed conclusions from the earlier animal cross-protection experiments, serum surveys in the area of origin and VP2 polymerase chain reaction (PCR) tests. We therefore conclude that the dominant BLU serotypes in China are BLU1 and BLU16. The isolate from Inner Mongolia might be BLU17, to be confirmed if antibodies are found in ruminants there.

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Table 1. Titration of Chinese isolates by microneutralisation test (titre of antisera that neutralised virus at $TCID_{50}$).

Isolates	Standard positive sera titres										
	1 Au[a]	1 SA[b]	3 SA	4	11	15 Au	16 SA	17 SA	20	23	Sx 1 sera
Sx2	64	80	–	–	–	–	–	–	–	–	20
Sx1	8	40	–	–	–	–	–	–	–	–	40
Yc	16	20	–	–	–	–	–	–	–	–	–
Y863P12	–	40	–	–	–	–	–	–	–	–	–
SWP9	–	–	20	–	–	–	80	–	–	–	–
WP7	–	–	20	–	–	–	80	–	–	–	–
NMP11	–	–	–	20	20	–	–	40	20	–	–
X27	–	20	–	–	–	–	–	–	–	–	–

[a]Au Australia antisera
[b]SA South African antisera
NB. All other antisera produced no neutralisation.

Regular Changes in Immunomorphology of Sheep Given Live Attenuated and Inactivated Bluetongue Vaccines

Chu Guifang, Wu Donglai, Yin Xunnan and Qu Xiangdong*

Abstract

Five groups of sheep were given inactivated bluetongue vaccine and four groups attenuated live virus vaccine, using 148 sheep in all. For these tests, Hubei inactive vaccine and Sichuan chicken embryo attenuated vaccine were chosen from nine vaccines as they were considered safe and effective. Inactive vaccine produced protection at 40 days post inoculation and attenuated vaccine at 20 days. The immune response to bluetongue disease was both humoral and cellular. Observations included immuno-histochemistry, histology, cellular chemistry and electron microscopy. No antigen was detected in the target cells of vaccinated sheep. T and B cells in the immune system and peripheral blood peaked at the same time. Sheep mounted an immune response immediately after challenge. No immune sheep challenged after vaccination had antigens in the target cells. Neither T nor B cells obviously increased or decreased immediately after challenge or as a delayed response.

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The Pathogenicity of Chinese Bluetongue Virus in Sheep, Deer and Goats

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Abstract

Buffalo, cattle and goats were inoculated with whole blood from diseased sheep which had been inoculated with the Yunnan strain of bluetongue virus serotype 1 (BLU1). Experimental animals showed a rise in temperature of about 0.5–1.2°C after 5–7 days and the number of leucocytes decreased by 30–50%. No clinical signs were observed. The same virus was used to inoculate musk deer (*Moschus moschiferus*), sika deer (*Cervus nippon*) and fallow deer (*Dama dama*). There was no rise in body temperature in these animals but the number of leucocytes decreased by 30–50% and some deer died 2–4 days after inoculation, although others survived. The main clinical signs included haemorrhages of the digestive tract, intestinal mucosa and lymph glands, liver and kidney. There was also necrosis and shedding of intestinal mucosa. Other clinical signs, observed in two deer, were haemorrhage and erosion of external nares plus shedding of mucosa in the nasal cavity. When inoculated into sheep, blood from these deer caused classic bluetongue disease.

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Identification of Monoclonal Antibody-defined Epitopes Validates the Bluetongue Competitive ELISA

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Abstract

The internationally recognised competitive enzyme linked immunosorbent assay (cELISA) for bluetongue relies on the ability of antibodies in sera to inhibit the binding of a mouse monoclonal antibody (MAb) to bluetongue virus (BLU) antigen. Suitable MAbs have been generated in several laboratories. MAb E9, in the Australian cELISA, reacts with the vast majority of BLU serotypes and its binding to BLU antigen is inhibited strongly by antisera to all Australian serotypes except BLU15. MAb E9 does not bind to BLU15. Mapping studies reveal that the E9 epitope lies between amino acids 30 and 38 of the major core protein VP7. Amino acids 21–54 are conserved in all sequenced VP7 genes and the location of epitope E9 validates use of MAbs to this region not only in cELISA but also as serogroup-specific reagents for virus detection. MAb E9 reacts with a hybrid molecule containing amino acids 1–76 of BLU15 and amino acids 77–349 of BLU1 VP7. Thus conformation of the E9 epitope depends on other parts of the molecule. The chimeric BLU1/BLU15 VP7 antigen will be tested for its ability to detect antibodies to BLU15. Field and experimental antisera to BLU compete with MAbs to the amino terminal domain of VP7. This part of the protein therefore is highly immunogenic and an ideal region with which to detect anti-BLU antibodies. Epitope topography in the amino-terminal portion of VP7 is retained in virus and core particles, baculovirus-expressed core and virus-like particles and in VP7 expressed in vaccinia and yeast. All such preparations may be used as antigens in the bluetongue cELISA.

ANTI-BLUETONGUE antibody in infected animals can be detected by a variety of tests. Complement fixation and agar gel immunodiffusion (AGID) tests (Boulanger and Frank 1975; Afshar et al. 1989) were popular before the development of a competitive enzyme linked immunosorbent assay (cELISA; Anderson 1984). The cELISA rapidly replaced the other two methods, which were cumbersome, reagent expensive and inadequate because they could not differentiate antibodies to BLU from those to other cross-reacting viruses (such as the epizootic hemorrhagic disease of deer viruses). The development of cELISA tests was made possible by the use of monoclonal antibodies (MAb) specific for BLU. While the first MAb-based cELISA was described by Anderson (1984), there have been several others since then, most notably those of Lunt et al. (1988) and Reddington et al. (1991).

From 1989 to 1992 several laboratories around the world compared the existing test protocols and reagents, the key variables in the tests being antigen and MAb. Antigens examined included virus and virus proteins in infected cell lysates (Anderson 1984; Lunt et al. 1988), sodium dodecyl sulfate-denatured viruses (Polkinghorne et al. 1992) and yeast-expressed (Martyn et al. 1990) and baculovirus-expressed (Oldfield et al. 1990) VP7 core protein. The trials involved laboratories in Australia, U.K., Canada and USA and the results (Afshar et al. 1992) were compared at a meeting in Vienna in October 1990 and presented at the bluetongue meeting at Office International des Epizooties, Paris in 1992

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(Jeggo et al. 1992). A summary of the method is given below.

The key reagent in cELISA is the MAb because it brings specificity to the test. Examples of MAbs used in cELISA are 3-17-A3 (Anderson 1984) and 20E9 (Lunt et al. 1988). In the cELISA, antibodies in test sera compete with the MAb for binding to antigen. In this paper we describe experiments to map the epitope to which MAb 20E9 binds. Our results validate the use of this, and other similar MAbs, in a cELISA for bluetongue.

cELISA Methodology

The following procedure for cELISA has been standardised after comparative studies in several international laboratories. Microtitre plates (96-well) are coated at 4°C overnight or 37°C for 1 hour, with 50–100 mL of antigen (tissue culture-derived sonicated virus (Anderson 1984); SDS-treated virus (Polkinghorne et al. 1992); or recombinant VP7 (Oldfield et al. 1990; Martyn et al. 1990) diluted in 0.05 M carbonate buffer, pH 9.6).

Plates are washed five times with PBST (0.01 M phosphate buffered saline containing 0.05% or 0.1% Tween 20, pH 7.2). Test sera are added (50 mL) in duplicate at a single dilution, either 1/5 (Afshar et al. 1989) or 1/10 (Lunt et al. 1988) in PBST containing 3% bovine serum albumen (BSA). Immediately into each well is placed 50 mL of a predetermined dilution of MAb diluted in PBST-BSA. MAb control wells contain diluent buffer in place of test sera. Plates are incubated for 1 hour at 37°C, or 3 hours at 25°C, with continuous shaking. After washing, wells are filled with 100 mL of an appropriate dilution of horseradish peroxidase-labelled rabbit anti-mouse IgG in PBST containing 2% normal bovine serum. Following incubation for 1 hour at 37°C, the conjugate solution is discarded and plates washed five times using phosphate buffered saline (PBS) without Tween. Wells are filled with 100 mL substrate solution containing 1.0 mM ABTS-4 mM H_2O_2 in 50 mM sodium citrate, pH 4.0 and plates shaken at 25°C for 30 minutes. (Other substrates may be used and the reaction continued with shaking for an appropriate time to permit colour development.) The reaction is stopped by adding a stopping reagent such as sodium azide.

After blanking the ELISA reader on wells containing substrate and stopper, the optical density (OD) values are measured at 414 nm. Results, expressed as percent inhibition, are derived from the mean OD values for each sample using the formula

$$\% \text{ inhibition} = 100 - [\text{Mean OD test sample} / \text{Mean OD MAb control}] \times 100.$$

Percentage inhibition values greater than 40% or 50% are considered positive. Results of test sera duplicates may vary as long as they do not lie either side of the chosen inhibition value. On each plate, strong and weak positive sera and a negative serum should be included. The weak positive should give 60–80% inhibition and the negative less than 40%.

Results

MAb E9, raised in mice against SDS-denatured BLU1, reacts in ELISA with yeast and vaccinia virus-expressed VP7 of BLU1 and with lysates of cells infected with all serotypes of BLU except BLU15 (Eaton et al. 1991). VP7 is the major core protein of the BLU virus (Huismans and Van Dijk 1990). Immuno-gold electron microscopy indicates that MAb E9 binds to intact virus particles, indicating that a portion of the underlying core particle is accessible at the virus surface. The E9 epitope is conformational: although it survives denaturation of VP7 with SDS, it is destroyed by heat and cannot be detected by western blotting. It is sensitive to reduction (by dithiothreitol) and acetylation (by iodoacetamide), suggesting a location near the only disulfide bond linking cysteine residues at amino acids 15 and 65 (Eaton et al. 1991). MAb E9 reacted with the amino terminal half of the VP7 expressed in bacteria. Attempts to map its position using truncated VP7 peptides as fusion peptides in *E. coli* failed because the products were insoluble and SDS and heat, needed for solubilisation, destroyed the epitope (Eaton et al. 1991).

We have made considerable progress in recent years in mapping bluetongue virus epitopes particularly using phage display technology (Du Plessis et al. 1994; Wang et al. 1995). Three different approaches have been used to map the E9 epitope used in the cELISA (Wang et al. 1994b).

MAb E9 does not react with BLU15 VP7. However, a hybrid molecule containing amino acids 1–76 of BLU1 VP7 linked to amino acids 77–349 of BLU15 was capable of reacting with MAb E9. The hybrid gene was cloned into the T7 RNA polymerase-directed expression vector pET-5b and transcribed and translated in vitro. MAb E9 was added to the mix followed by immunobeads coated with rabbit anti-mouse antibody. The immuno-captured material was analysed on SDS gels. Chimeric BLU1/15 VP7, but not BLU15 VP7, was captured by MAb E9. Thus the E9 epitope is present in the first 76 amino acids of the VP7 molecule.

A second approach made use of another anti-VP7 MAb (1AA4, provided by Dr J. Meecham, ABADRL, Laramie, Wyoming, USA) which competed with MAb E9 for binding to yeast-expressed

VP7 of BLU1. Unlike MAb E9, MAb 1AA4 reacted with VP7 in western blots. Truncated VP7 peptides of BLU1 VP7 were made as C-terminal fusion proteins to pGEX expressed glutathione S-transferase. Western blots indicated that MAb 1AA4 reacted with the first 68 amino acids of VP7.

The third approach used epitope scanning by competition with short synthetic peptides. Five peptides were made which covered amino acids 11–29, 20–29, 30–47, 39–47 and 48–63 of BLU1. Binding of MAb E9 to yeast-expressed or viral VP7 was reduced by 87% by the peptide containing amino acids 30–47. The peptide containing amino acids 39 to 47 did not inhibit. These data suggest that the E9 epitope lies within amino acids 30–38. The peptide containing amino acids 48–63 inhibited MAb 1AA4 binding by 90%.

The region 39–47 is conserved across all BLU serotypes, which confirms the usefulness of a MAb which binds in that region as a serogroup-specific diagnostic reagent.

Discussion

Recent cryo-electron microscopic and X-ray crystallographic data have indicated that VP7 exists in virus core particles as a trimer (Basak et al. 1992; Grimes et al. 1995). Topographic studies (Hewat et al. 1992; Hyatt and Eaton 1988) have confirmed that the molecules are arranged with at least some of them having their amino termini accessible from the outside of core particles. Many MAbs generated to VP7 from several sources compete with MAb E9 and therefore bind to the exposed part of the VP7 trimer (Wang et al. 1992). As field ovine and bovine sera, and experimental rabbit, ovine, murine and bovine polyclonal antisera to all BLU serotypes contain antibodies that compete with MAbs to the amino terminal domain, this part of the protein appears highly immunogenic in all species tested.

The amino acid sequence 21 to 54 is absolutely conserved in all sequenced BLU VP7 genes, including BLU15 (Wang et al. 1994a) and the E9 epitope is located in the middle of this conserved region (Iwata et al. 1992). The failure of MAb E9 to bind to BLU15 VP7 suggests that the sequence of the binding site alone is not sufficient to provide a functional epitope for the MAb. Since the BLU1/15 VP7 chimera was able to bind MAb E9, residues close to the binding site are probably critical in maintaining conformation of a functional binding site.

Although the cELISA used at the Australian Animal Health Laboratory (AAHL) is robust and highly effective, it suffers one minor disadvantage in that it detects anti-BLU15 antibodies with low efficiency, presumably because antisera to BLU15 lack antibodies to the E9 epitope. The failure of cELISA to detect antibodies to all BLU serotypes with high efficiency is not unique to the AAHL test. In the cELISA described by Anderson (1984), antibodies to BLU19 were detected with lower efficiency than those to other serotypes. Current data suggest that the efficiency of detection of anti-BLU15 antibodies in our cELISA can be improved to some extent if antigen preparations other than yeast-expressed VP7 are used in the test. The ability of anti-BLU15 antiserum to compete with MAb E9, albeit weakly, may be due to the fact that, although the E9 epitope is not shared between BLU15 and the BLU1 antigen used in the test, these viruses may have other epitopes in common. Antibodies to shared epitopes in BLU15 antisera may be able to inhibit sterically the binding of MAb E9 to BLU1 antigen. We are currently investigating the possibility of using the chimeric BLU1/15 VP7 molecule as an antigen in the cELISA.

At present, the greatest need in bluetongue diagnosis worldwide is for alternative methods to determine the serotype specificity of antibodies in test sera. Currently, serotype-specific antibodies are detected in neutralisation tests which are labour intensive, time consuming, unsuitable for analysis of large numbers of sera and which, because live virus is used, may have to be done in secure laboratories. At first sight an attractive option would be to develop cELISA procedures in which antibodies in test sera compete with serotype-specific MAbs for binding to antigen. The difficulty with such an approach is the requirement that the MAb must bind only to its homologous serotype. Unfortunately, the extreme paucity of MAbs that display serotype-specific binding suggests that it will be difficult to generate MAbs that bind to only one serotype. Indeed, MAbs that neutralise one serotype may not only neutralise other serotypes but may also bind to, and not neutralise, many heterologous serotypes (Ristow et al. 1988; White and Eaton 1990).

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Comparison of Competitive ELISA and Agar Gel Immunodiffusion Tests to Detect Bluetongue Antibodies in Ruminants in China

Ben Jin*, Li Zhihua*, Li Huachun*, L.F. Melville†, N.T. Hunt†, Li Xinrong*, Zhang Fuqiang* and Zhang Nianzu*[1]

Abstract

The agar gel immunodiffusion test (AGID) has been widely used as a bluetongue group antigen test. The competitive enzyme linked immunosorbent assay (cELISA) has been established for a few years and has become a recognised technique for the same purpose. Serum samples tested by AGID were also tested by cELISA. The capture antigen for the cELISA was the expressed yeast VP7 of BLU1. The AGID used antigen prepared from BLU1 isolated in Yunnan. The cELISA test used a monoclonal antibody competing with positive serum to bind the VP7. The AGID test used a reference serum produced in sheep by inoculation of homologous BLU1. Tests of 560 samples from goats, sheep and cattle showed that 85.4% of results were identical: however, 75 sera that showed positive in the cELISA were negative in the AGID, while seven samples positive in the AGID were negative in the cELISA. These results suggest that the cELISA may be more sensitive than AGID in the detection of bluetongue antibodies.

AGAR gel immunodiffusion (AGID) was the earliest method used in China for serological surveys of bluetongue antibody. Zhang Nianzu et al. (1989c) first found bluetongue disease in China in Yunnan in 1979 and developed the AGID to study bluetongue (BLU) epidemiology (Zhang Nianzu et al. 1989b). The first use of the enzyme linked immunosorbent assay (ELISA) for bluetongue research in China was at the Institute of Animal Quarantine, Ministry of Agriculture, Qingdao. The ELISA was also used at the Yunnan Institute of Animal Husbandry and Veterinary Medicine to select monoclonal antibody (MAb) for BLU research by Wu Dexing and Zhang Khaili (1991). To improve the sensitivity and specificity of the test for BLU, a competitive ELISA (cELISA) was developed using a MAb for the BLU group-specific antigen VP7.

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Materials and Methods

Five-hundred-and-sixty serum samples were tested from goats, sheep and cattle. Some of these were collected as part of a serological survey and some were from sentinel herds in Yunnan (Fig. 1) established by Li Huachun et al. (these Proceedings) and others from Inner Mongolia (Fig. 2).

The cELISA method was based on the method of Lunt et al. (1988) and Martyn et al. (1990). Tests were performed with TropBio ELISA Bluetongue Kits (Tropical Biotechnology Pty Ltd, James Cook University, Townsville, Australia).

The soluble antigen and reference sera were made as described by Zhang Nianzu et al. (1989a). Procedures for the AGID tests for BLU-antibody followed those established by Yunnan Institute of Animal Husbandry and Veterinary Medicine (Anon. 1989).

Results

One-hundred-and thirty-nine samples were positive in both tests, while 339 samples were negative in both tests (Table 1). In the cELISA, 214 (38.2%) samples were positive while 146 (24.1%) samples

were positive in the AGID test. Different results in the two tests occurred for 82 samples: 75 were positive in the cELISA but negative by AGID while seven samples were positive in the AGID but negative by cELISA.

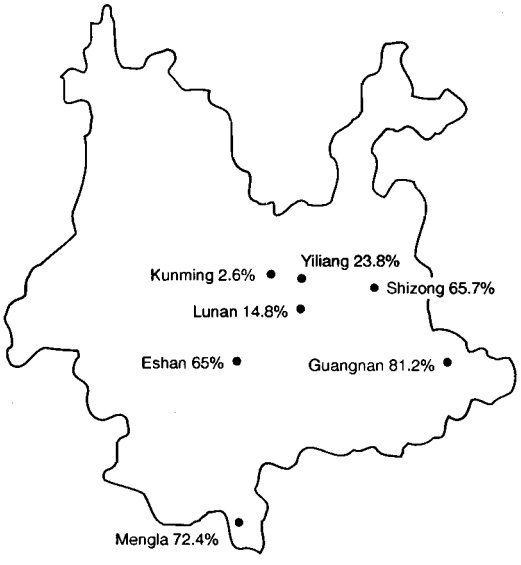

Figure 1. The cELISA positive rate of BLU-antibody in Yunnan.

The cELISA positive rate for sera was 21/29 from Mengla; 56/69 from Guangnan;. 21/56 from Kunming; 19/80 from Yiliang; 12/81 from Lunan; 23/35, from Shizong; and 52/80 from Eshan. In Yunnan, the cELISA positive rate for cattle was 177/273 while the rate for sheep and goats was 10/257. In Inner Mongolia, 27 of 30 goats were cELISA positive. Some of these results are summarised in Table 2.

Table 1. Comparison of cELISA/AGID serology.

	cELISA (+)	cELISA (–)	Total
AGID (+)	139	7	146
AGID (–)	75	339	414
Total	214	346	560

Discussion

This study suggests that the cELISA is more sensitive than AGID in detecting bluetongue antibodies. The major core-protein of BLU, VP7, is the serogroup-specific antigen. The binding of monoclonal antibody with VP7 is inhibited by a small amount of antibody to bluetongue viruses in the sera. Detection of antibody binding to VP7 also reduces the possibility of non-specific reaction giving a more specific result with the cELISA than the AGID test.

Animals infected by BLU produce antibodies which decline over time unless the animals are reinfected with another serotype. The reduced concentration of antibody may be insufficient for detection by AGID but may be detected by the more sensitive cELISA. This may explain why 75 samples were positive in cELISA but negative in AGID.

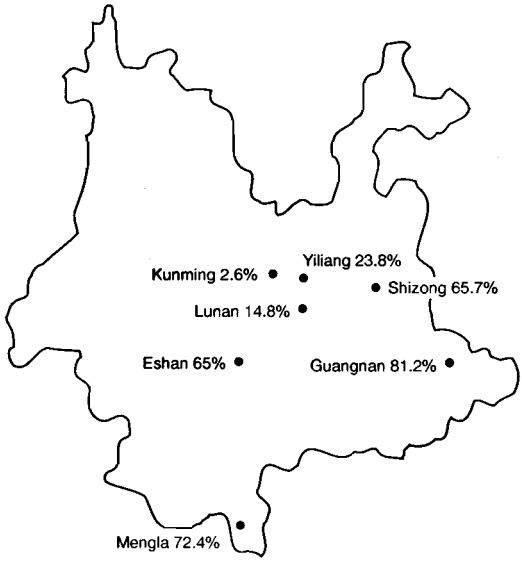

Figure 2. The sites of samples collected in China.

Table 2. Comparison of cELISA/AGID results on samples collected from 1992–95.

Place	Animals	AGID+	cELISA+	AGID+/ cELISA–	cELISA+/ AGID–	AGID+/ cELISA+
Yiliang	goats	2/40	2/40	0	0	2
	cattle	10/40	17/40	1	8	9
Lunan	goats	2/61	3/61	0	1	2
	cattle	1/20	9/20	0	8	1
Shizong	goats	0/5	0/5	0	0	0
	cattle	14/30	23/30	1	10	13
Mengla	cattle	14/29	21/29	1	8	13

Orbiviruses, such as BLU and epizootic hemorrhagic disease virus (EHD), have group-antigens in common, and so can stimulate the host to produce antibodies which may be detected by the AGID for BLU antibody. The seven samples positive in the AGID but negative in the cELISA may be the result of infection with other orbiviruses.

The sera from Mengla and Guangnan (Fig. 1) showed high levels of BLU antibody. Mengla and Guangnan lie in the south and southeast of Yunnan respectively, in typical tropical and subtropical regions. In summer and autumn, the humid and hot climate is suitable for the maintenance of *Culicoides* midges, the BLU vector. In Kunming, 300 km from Guangnan and 500 km from Mengla, few ruminants have antibodies to bluetongue, suggesting little vector activity in Kunming. The geographic distribution of other survey and sentinel sites indicates a general decrease in prevalence from south to north and with increasing altitude.

In Yunnan Province, the cELISA positive rate was higher in cattle than in sheep or goats, indicating a possible vector preference for cattle. In contrast, the cELISA positive rate was 27/30 in goat samples from Inner Mongolia, while the serological survey results from the local veterinary station show very few cattle with BLU antibody. This may indicate a different preference by vectors in Inner Mongolia, or a different vector entirely.

As the seven samples that were AGID positive and cELISA negative included one goat from Inner Mongolia and six cattle from Yunnan, infection with other related orbiviruses may be more likely to occur in cattle than in sheep or goats.

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Detection of Bluetongue in China by Polymerase Chain Reaction (PCR)

Peng Kegao*, A.R. Gould†, Li Huachun*, Ben Jin*, J.A. Kattenbelt†, Li Xinrong*and Zhang Fuqiang*

Abstract

The polymerase chain reaction (PCR) was used to confirm the presence of bluetongue virus (BLU) in tissue culture cells, chicken embryo and clinical blood samples. BLU VP3 specific oligonucleotides were used in PCR-based diagnostic tests to exclude the possibility of other orbiviruses, which corroborated previous field and diagnostic assessments. The VP3 oligonucleotides, which successfully amplified genes sequences from the BLU isolates from China, have been successfully tested previously on viral isolates from many temperate zone regions around the world. A set of oligonucleotides with sequences derived from the Australian BLU1 VP2 sequence was tested for its ability to act as a set of 'generic' VP2 primers to amplify VP2 sequences irrespective of viral serotype. From an analysis of these VP2 PCR reactions, we were able to determine that a minimum of three separate serotypes were present in the isolates tested. PCR analysis of blood samples from Shanxi, performed in 36 hours, demonstrated the speed and efficiency with which a positive diagnosis could be made, and compared very favourably with more traditional diagnostic and tissue culture methods for identifying viruses in sera.

BLUETONGUE virus (BLU) is the type member of the orbivirus genus within the family Reoviridae. BLU possesses a segmented genome of 10 double-stranded (ds) RNA genes which code for seven structural and three non-structural proteins within virus-infected cells. Many attempts have been made to construct gene probes (Purdey et al. 1984; Ghiasi et. al. 1985; Squire et al. 1985; Mertens et. al. 1987; Pedley et al. 1988; Gould 1989; Wilson 1990) or polymerase chain reaction (PCR) tests (Wade-Evans et al. 1990; Dangler et al. 1990; Wilson et al. 1990; McColl and Gould 1991, 1994) to identify and discriminate this serogroup from other orbiviruses. However, the variation of the gene segments for BLU isolates from different regions of the world (Gould and Pritchard 1990, 1991; Pritchard and Gould 1995) have made this approach untenable in certain situations. VP3, VP7 and NS1 gene probes vary by as much as 20% at the gene level within the same serogroup, and by 30% when compared to other closely related orbiviruses (Gould 1987, 1988; Gould et al. 1988; Gould and Pritchard 1991). Using a gene probe to discriminate between closely related orbiviruses and bluetongue viruses from different geographic regions, by varying the hybridisation conditions, has thus proven unreliable (Gould 1987). While polymerase chain reaction (PCR) tests have been used to identify BLU isolates in clinical samples and from different viral isolates, in general these oligonucleotides have been designed to amplify gene sequences from the same geographic region (Wade-Evans et al. 1990; Dangler et al. 1990). Only one set of oligonucleotides has been rigorously tested on BLU isolates from different geographic regions (McColl and Gould 1991; Gould and Pritchard 1991). These oligonucleotides have been used successfully to amplify BLU isolates from Australia, Indonesia, Malaysia, India, South Africa, the Caribbean and North America (Pritchard and Gould 1995). In this way, three major and several minor geographically distinct types of viruses (topotypes) have been identified. The speed and accuracy

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of the BLU PCR has been demonstrated adequately by several laboratories. However, to be a truly universal system for the rapid identification of BLU viruses, these diagnostic oligonucleotides must be capable of amplifying a gene segment irrespective of the topotype of the BLU isolate. To this end we have attempted to test the response of the BLU PCR to virus present in tissue culture cells, chicken embryos and clinical blood samples of BLU isolates in China.

Materials and Methods

Viral isolates

In this study the viral strains came from isolates held at the Yunnan Tropical and Subtropical Animal Laboratory, Jindian, Kunming. Unless stated otherwise, viruses were isolated from sheep blood by direct inoculation onto BHK cells. BLU strains used in this study were:

- BLU-H originally isolated in Wuhan province (Hubei) in 1987;
- BLU-IM8 and BLU-IM isolated from goats in Inner Mongolia in 1988 and 1990 respectively;
- BLU-Y isolated in Yunnan (Jindian province) in 1979;
- BLU-XJ isolated at Xinjiang in 1989; and
- BLU-SX, sheep blood from a suspected BLU outbreak at Shangxi province in October 1993.

BLU-Y was inoculated into a chicken embryo: after 72 hours, the brain, heart, liver and kidney were dissected, homogenised using a mortar and pestle, and the nucleic acids extracted as described by McColl et al. (1994).

DNA oligonucleotides and PCR conditions

DNA deoxyoligonucleotides were synthesised on an Applied Biosystems DNA synthesiser using phosphoramidite chemistry. Oligonucleotides were purified either by electrophoresis through 14% polyacrylamide gels followed by elution and C_{18} Sep-Pac column elution, or by precipitation using N-butanol (Sawadogo and Van Dyke 1990). The oligonucleotides for both VP3- and VP2-based PCR reactions (McColl and Gould 1994; Pritchard and Gould 1995) were used to amplify BLU-specific sequences from nucleic acids extracted from tissue culture cells, chicken embryos and clinical blood samples. PCR conditions were as described by McColl and Gould (1994) for both primary and nested (secondary) amplification cycles. PCR products were visualised in 1% agarose-tris-acetate-EDTA gels after electrophoresis in the presence of ethidium bromide and illumination with ultraviolet light. DNA markers were prepared by the digestion of DNA with the restriction endonuclease AvaII following the manufacturer's instructions.

Results

Nucleic acids extracted from BLU-infected BHK cells were subjected to amplification using PCR and BLU VP3-specific oligonucleotides. BHK cells uninfected with virus were used as controls. Of the strains tested, seven were positive in BLU-specific reactions, while two were consistently negative (Table 1). The latter were an unknown virus isolated from insects (a suspected BLU designated WT-I) and BHK control cells. Of the isolates positively identified as BLU by VP3-specific PCR tests, four were also found to be positive in PCR reactions using the 'generic' VP2-specific primers, again confirming the BLU identity of these isolates. Two of the BLU isolates (BLU-H and BLU-S) were consistently negative in both primary and nested PCR tests. BLU-Y, which gave a strong PCR product using the primary VP2 primers, produced a slightly weaker positive signal after nested PCR. The ability of the PCR tests to detect low levels of BLU circulating in chicken embryo tissues was also tested (Table 1). Embryo brain tissue was a rich source of virus, while the other tested tissues had lower levels of virus. A clinical sample was also included to demonstrate the applicability of the PCR test to detect virus circulating in sheep's blood. Using a nested PCR, BLU-S was identified as being present in the clinical sample, although the amount of virus in this sample was insufficient to be detected in a primary PCR reaction. This occurrence has been observed routinely in clinical blood samples tested for the presence of BLU (McColl and Gould 1994).

Discussion

This study has indicated the usefulness of PCR as a diagnostic technique. The results reported here demonstrate that PCR can discriminate between BLU and other viruses, and that the level of non-specific reactions from tissue culture cells and clinical samples is negligible. We found that PCR tests were able rapidly to detect the presence of virus in clinical blood samples from infected sheep, without previous amplification using tissue culture or egg inoculation. This greatly reduces the time needed for a positive diagnosis from a matter of weeks to 36 hours. The sensitivity and specificity of the PCR test has been investigated thoroughly (McColl and Gould 1991, 1994; McColl et al. 1994). The ability to detect virus present in the tissues of a chicken embryo demonstrate the usefulness of the technique to detect the presence of high titre virus in tissues before attempted virus propagation through tissue culture. Again this should decrease the time taken for virus identification and characterisation.

Sequencing PCR products can generate valuable information about the phylogenetic relationships and serotypes of these viruses (Gould and Pritchard 1990, 1991). The differential reaction of the Chinese BLU isolates with the VP2-specific serotypes in primary and nested PCR tests indicated that there were at least three different serotypes in China. Serotype determination will have to await either sequencing studies or serum neutralisation tests. PCR-based tests have also shown that the primers used in these studies have universal application in the detection and identification of viruses of the bluetongue serogroup, irrespective of their geographic origin. To date these are the only 'serogrouping' oligonucleotides thoroughly tested on BLU isolates from around the world using clinical, tissue culture, blood or fixed tissues in PCR.

Table 1. Reactions of Chinese viral isolates with serogroup and serotype-specific BLU primers.

BLU isolate[a]	Primers	
	VP3	VP2
H	+++ (+++)	—
S	+++ (+++)	—
IM	+++ (+++)	+++ (+++)
1M-8	+++ (+++)	+++ (+++)
Y	+++ (+++)	+++ (++)
SX	(+++)	nt
XJ	+++ (+++)	+++
WT-I	—	—
BHK control nucleic acid	—	—
Brain[b]	+++	nt
Kidney[b]	++	nt
Heart[b]	+	nt
Liver[b]	+	nt

[a] see text for descriptions of isolates
[b] nucleic acid isolated from chick embryo tissue
\+ denotes positive reaction after PCR
++ denotes strong positive reaction after PCR
+++ denotes very strong positive reaction after PCR
— denotes no observable amplification product after PCR
( ) denotes the strength of the amplification signal observed after nested PCR
nt not tested

Acknowledgments

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Studies of a Non-radioactive Gene Probe for Bluetongue Viruses

Peng Kegao, Li Zhihua and Li Gen*

Abstract

A non-radioactive gene probe was used for bluetongue virus (BLU) analysis. RNA was extracted and purified, at the peak of the cytopathic effects (CPE), from BHK21 cell cultures inoculated with Yunnan local isolate. This RNA was identified as bluetongue (OD260/OD280 was > 1.6). It was then labelled with non-radioactive labels including biotin and photobiotin. The Dot-Blot technique was used to test the probe both with biotin-AKP and HRP. The non-radioactive labelled RNA probe was obviously hybridised with known bluetongue viruses and not with epizootic hemorrhagic disease (EHD) virus, cell RNA or the negative control. The background of biotin-AKP was lower than that of biotin-HRP. The technique using a non-radioactive gene probe to study bluetongue appears acceptable, as it can distinguish bluetongue group specificity, although not individual serotypes. In comparison with the radioactive gene probe, the non-radioactive labelled BLU was easily, safely and effectively applied for analysis of bluetongue viruses.

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Analysis of Local Isolates of Bluetongue Viruses in China by SDS-PAGE

Hu Yuling and Peng Kegao*

Abstract

The RNA of four bluetongue isolates from Yunnan, Hubei, Sichuan and Inner Mongolia (China), and of two standard serotypes from USA (BLU-A10 and BLU-A17), were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). All six samples were prepared from BHK21 cell cultures showing cytopathic effects (CPE) after inoculation with the isolates and serotypes. Ten fragments were seen on the gels of all samples, which could be divided into four groups: 1–3, 4–6, 7–9 and 10. The type of fragments of RNA was 3, 3, 3, 1. There were no significant differences between the types of fragments from local isolates and those from standard serotypes. Thus SDS-PAGE used in this way could be a technique for bluetongue group identification but would not be recommended as the method for identification of a specific serotype.

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A Study of the ELISA Test for the Detection of Epizootic Hemorrhagic Disease (EHD) Virus Infection

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Abstract

New Jersey-EHD1, Canadian Alberta-EHD2 and Ibaraki viruses were grown in BHK21 cells and purified as antigens for enzyme linked immunosorbent assay (ELISA). Antigen prepared with Ibaraki virus gave quite good group-specificity. The whole cell cultures were centrifuged and the supernatant, with 0.5% NP_{40} added and treated with 250 units/mL heparin, subjected to ultracentrifugation. The purified viral particles as antigen gave the best results. Based on the agar gel immunodiffusion (AGID) results from 1420 cattle and sheep sera, the upper limit of the 99% negative confidence was 2.5 and the lower limit of the 99% positive confidence was 3.0, estimated by statistical treatment of the ratio of sera test value/standard negative sera test value with ELISA. The zone between 2.5 and 3.0 was regarded as suspicious. All 2484 cattle and sheep sera submitted for examination, tested by both AGID and ELISA, had a positive rate of 19.04% with AGID and 26.037% with ELISA. Samples that tested positive by ELISA included 99.58% of the samples that tested positive by AGID. When sera from a further 4296 cattle and sheep were tested by ELISA, the positive rate was 22.94%.

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A Rapid ELISA Procedure for Detection of Antibody to Bluetongue Viruses

Yang Chengyu, Cheng Xiengfu, Ma Hongchao, Fan Gencheng, Li Xiaocheng and Pu Shuying*

Abstract

By optimising the reaction conditions, a rapid enzyme linked immunosorbent assay (ELISA) procedure was developed to detect bluetongue antibodies. After the coating process the procedure was completed in 45 minutes, two thirds of the previous required time, with the same sensitivity. Using this process to test 70 sera resulted in 12.9% more positives being detected than with agar gel immunodiffusion (AGID).

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A Serological Identification of Shandong Strain of Bluetongue Virus

Yang Chengyu, Pu Shuying, Ma Hongchao, Cheng Xiengfu, Li Xiaocheng, Fan Gencheng and Zhang Yianxia*

Abstract

An isolate (L001) of bluetongue virus was typed using the plaque inhibition test with both standard type-specific antisera and those prepared by this laboratory. A suspension procedure of plaque inhibition was developed to type the isolate. The results showed the isolate was identical to the standard strain of BLU16. In addition, the RNA-map of L001 in polyacrylamide gel electrophoresis (PAGE, 10%) was the same as that of BLU16, confirming that L001 was BLU16.

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Serological Cross-reactions between Bluetongue and Epizootic Hemorrhagic Disease (EHD) Virus Groups in Agar Gel Immunodiffusion (AGID) Tests

Li Xiaocheng, Yang Chengyu, Chang Yanxia, Pu Shuying and Cheng Xiengfu*

Abstract

Antisera and antigens were prepared with bluetongue (serotypes BLU1 to BLU22), epizootic hemorrhagic disease (EHD1, 2) and Ibaraki viruses. Agar gel immunodiffusion (AGID) tests gave the following results:

- no reaction occurred between EHD2 antigen and EHD1 antiserum
- precipitin lines developed between antigens of EHD1 or Ibaraki virus and antiserum to EHD2
- antigens to BLU10, 17 and 20 reacted with antisera to EHD1, 2 and Ibaraki viruses
- EHD1 antigen reacted with antisera to BLU4, 9, 10, 12, 14, 15, 17 and 20
- EHD2 antigen reacted with antisera to BLU4, 10, 12, 16, 17, 20 and 21
- no cross-reaction occurred between Ibaraki antigen and antisera to BLU1 to BLU22
- cross-reactions between EHD antigens and antisera to BLU1 to BLU20 were related to repeat inoculations of animals with single BLU serotypes
- EHD1 antigen did not react with antisera to BLU16, 18, 19, 21 and 22 from animals which had received a single dose in their preparation, but did react after second doses
- EHD2 antigen did not react with antisera to BLU14, 15, 18, 19 and 20 from animals which had received a single dose in their preparation, but did react after second doses.

There is thus ample evidence of cross-reactivity between antigens prepared from EHD virus and antisera to bluetongue serotypes.

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A Comparison of BHK21, Vero and C6/36 Cell Lines in the Preparation of the AGID Antigen of Bluetongue Virus

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Abstract

Agar gel immunodiffusion (AGID) is a test for detecting bluetongue virus commonly used in some regions and countries. Years of comparative experiments have shown the C6/36 cell line to be very much superior to BHK21 and Vero cells in the preparation of the AGID antigen of bluetongue virus serotype. Antigen production in C6/36 was 25 times greater than with BHK21 or Vero cells. Antigen titre with BHK21 or Vero was <1:8 while the titre with C6/36 was >1:8, and AGID with BHK21 or Vero antigens could be read in 48 hours. Parallel and block tests showed both antigens had equal specificity. The C6/36 antigen revealed clear and dense precipitating lines with positive sera.

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Enhancement of Growth of Bluetongue Viruses in Cell Cultures through Trypsin Treatment

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Abstract

Bluetongue viruses were treated with trypsin (final concentration 5μg/mL) for 30 minutes at 37°C, then inoculated onto BHK21 or Vero tissue culture monolayers. The maintenance medium contained trypsin at 1 μg/mL. This method promoted viral propagation, enhancing the titre from $10^{4.62}$ to 10^{7} in BHK21 cells and from 10^{4} to 10^{6} in Vero cells. The treated viruses were passaged in cell culture so viability was not affected. Polyacrylamide gel electrophoresis (PAGE) indicated that the virus protein structure had not changed.

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Comparison of the Value of AGID and ELISA Antigen in the ELISA Test for Bluetongue Viruses

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Abstract

Sera from 125 cattle and sheep in a bluetongue epidemic area, and 3020 survey sera, were tested in parallel using agar gel immunodiffusion (AGID) or enzyme linked immunosorbent assay (ELISA) tests with ELISA antigen. The tests matched for quality and quantity, and the results showed a high correlation; 0.998 for cattle sera and 0.999 for sheep sera. The similarity of these results lead us to suggest that AGID antigens could be used instead of ELISA antigens.

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Vaccine and Control

Introduction

IN this section the spectrum of vaccines is canvassed. Vaccines used in sheep on farms have ranged from empirical applications, without knowing the relevant serotype identity, to the use of inactivated whole virus, or inactivated and attenuated live virus. Non-living bluetongue virus constructs (for example, core-like particles, or CLPs) have been shown to be effective on an experimental basis. With the classification of all available bluetongue strains, the need for empirically developed vaccines has now passed in China. All countries in this region can now have their bluetongue viruses identified to serotype. Information on serotypes known to exist in Southeast Asia and the Pacific countries is increasing rapidly. This means significant problems in having available vaccines for many of the serotypes, a situation that applies regardless of whether manufacture is of safe inactivated vaccines or of the controversial, teratogenic, live attenuated vaccines.

Vaccines made from artificial constructs of bluetongue viruses can be made polyvalent, to a limited extent, and are safe. Papers in this section describing the value of core-like particles in reducing the severity of bluetongue disease in sheep suggest broad spectrum effectiveness. Certainly, a broad spectrum vaccine would not only limit the economic effects of outbreaks but would also allow preconditioning of susceptible sheep that were to be imported into endemic areas. Knowing the threatening serotype would then become of secondary importance. Further testing of core-like particles as vaccines seems very important in countries of this region.

The situation that has evolved in the United States of America and South Africa may have been partially created by the intense use of live attenuated vaccines, which made available genes for transfer to naturally occurring viruses. The altered genotype may enable the virus to cross the placenta and damage the foetus. Once released into the environment, altered bluetongue live viruses may exchange genetic information by reassortment in cattle, sheep, goats, deer or *Culicoides*. There is still a chance of avoiding this fundamental mistake in Asia and Australia.