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Studies on the epidemiology of bluetongue virus in China

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SUMMARY

Sentinel herds of large ruminants were established at five centres in Yunnan Province, Peoples Republic of China, between 1995 and 1997. The application of a sensitive antigen capture ELISA to facilitate virus isolation procedures led to the isolation of 108 strains of bluetongue (BLU) virus. Serotypes isolated included types 1, 2, 3, 4, 9, 11, 12, 15, 16, 21 and 23. Virus transmission occurred over a period of 1–3 months at each of the four positive sites, giving an overall BLU virus transmission period for the province of 5 months, from early June to early November. The greatest level of transmission took place in July and August. The duration of viraemia in individual animals varied from 1 to 7 weeks, with a mean calculated for each serotype between 6 and 20 days. The study represents the first detailed investigation of the epidemiology of BLU in China utilizing sentinel herds.

INTRODUCTION

For some time bluetongue (BLU) has been recognized as an important disease of sheep in China, but the range of serotypes in the country and their epidemiology has been largely unknown [1]. In 1994, a collaborative study commenced between Chinese and Australian scientists. The project was centred in Yunnan Province in the south west of the country, with the aims of determining the serotypes of bluetongue within that province and elucidating their epidemiology. These objectives were met by the establishment of herds of sentinel cattle at several sites within the province and monitoring them serologically and virologically over several years. Limited cross-sectional serological studies were carried out on samples from other locations in Yunnan, and also in some other provinces. A brief summary of some of this work has been reported [2]; the current paper describes the epidemiological aspects in detail. Later studies [3] report the serological and molecular characterization of some of the viruses isolated.

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MATERIALS AND METHODS

Sentinel herds

Herds were established progressively from 1994, in five districts (Fig. 1).

The Kunming herd was established in late 1994 in Shuanglong village, 30 km north of the provincial capital Kunming, approx. 2000 m above sea level. In

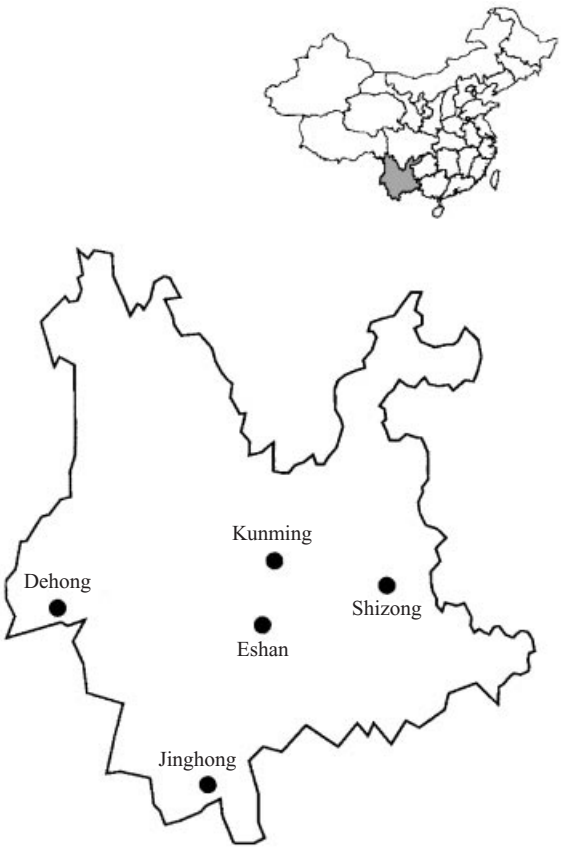

Fig. 1. Location of sentinel herds in Yunnan Province, P.R. China.

1995, the Shizong county herd was located in Wulong village, 260 km to the east of Kunming, at an altitude of 1600 m and the Eshan country herd was sited at Baoqian village, 150 km south of Kunming, at an altitude of 1400 m. Additional herds were set up at Jinghong and Dehong in 1996. Jinghong is the regional centre of Xishuanbanna district, situated near the southern border of Yunnan Province, about 400 km south of Kunming. The area is at a lower altitude (400–1000 m) and is distinctly tropical, in contrast to the other herd sites farther north. The Dehong district herd was situated in the village of Mangshi, about 400 km from Kunming also at an elevation of 400–1000 m in a subtropical environment towards the western border.

Sentinel herds consisted of groups of 10–20 local animals (cattle, buffalo, and in one instance (Kunming), also goats) which showed no evidence of prior bluetongue infection by testing for group antibodies by the blocking ELISA (bELISA). Sometimes, initial screening for the selection of animals was undertaken locally, using the agar gel immunodiffusion (AGID) test. Where insufficient seronegative animals were available, a few positive animals were included on the assumption that they may have been infected by only a limited number of serotypes and were probably susceptible to most other serotypes. At approximately annual intervals, animals that had become infected during the previous year were replaced by new seronegative animals.

Blood was collected into both heparinized (for virus isolation) and plain evacuated tubes (serology) at weekly intervals during the warm months of the year, and at monthly intervals during the cooler months. (At Shuanlong, where no seroconversions were recorded in 1994/5, monitoring frequency was reduced to monthly intervals for the latter part of the study.) Samples were despatched to the Yunnan Tropical and Subtropical Animal Virus Diseases Laboratory (YTSAVDL), Kunming, where all laboratory procedures were performed.

LABORATORY METHODS

Serology

Bluetongue group-reactive antibodies

The bELISA and AGID tests were performed as described previously [4] using cell culture antigen derived from BLU1 for both tests. In the bELISA, the antigen-coated plates were treated with a commercial stabilizer to facilitate long term usage, and the Mab 8A3B6 [5] was used as the competing anti-VP7 monoclonal antibody.

Type-specific antibodies

Reference strains of BLU virus serotypes 1–24, were obtained from the OIE Reference Laboratory for Bluetongue and African Horsesickness, Onderstepoort Veterinary Research Institute (OVRI), Republic of South Africa, and passaged in BHK21 cells. Type-specific antibodies against these viruses were detected by means of a virus neutralization test (VNT) using Vero or BHK21 cells in a 96-well microplate system as described (4). Not all sera were tested with all serotypes. The serotypes that were used were prioritized on the basis of the knowledge of their presence in China at the time, and the likelihood of their presence in China and the surrounding regions [6].

Virus isolation and identification

Virus isolation was undertaken strategically, relative to seroconversion in the bELISA. When an animal

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seroconverted, the blood samples for the previous 2 weeks, the week of seroconversion and all subsequent samples were processed until there was clear evidence of a cessation of the presence of virus in the animal. The preparation of heparinized blood for intravenous inoculation into embryonated chicken eggs (ECEs) and the subsequent incubation, harvesting and processing of chicken embryo tissues were performed as described previously [7].

In place of the usual regime for BLU virus isolation (intravenous inoculation of ECEs, and passage of ECE tissue in insect and mammalian cell culture), embryo tissues were screened for BLU antigen in a sensitive BLU antigen capture ELISA (BTACE) [8]. Positive liver samples were further processed for virus isolation by one passage in insect (*Aedes albopictus* C6/36) and up to three passages in mammalian (BHK-21) cell cultures. Viruses isolated in cell culture were identified to BLU group level in an immunoperoxidase test (4) using a mixture of group reactive monoclonal antibodies (7D3A2/8A3B6) [5]. Viruses giving positive results in this test were identified to serotype level in a microtitre neutralization test using Vero cells and reference type-specific antisera obtained from the OIE Reference Laboratory for Bluetongue and African Horsesickness, OVRI, Republic of South Africa.

These methods were also used to identify the eight viruses isolated over the previous 15 years [1, 9].

Serological studies

During the study, 5427 sera were collected for serology. The majority (3621) were from sentinel herds in Yunnan province while others came from collections made for other purposes (1281 from Yunnan and 525 from other regions (Hubei, Inner Mongolia, Shanshi, Sichuan, and Xinjiang)).

Calculation of duration of viraemias in sentinel herds

The precision of estimates of the duration of viraemias is governed by the frequency of sampling (in this study, weekly). In some instances, viruses were only isolated on a single day. In theory, these animals may have been infected up to 6 days both before and after that day, without virus being detected at a second sampling point. For the purpose of estimating the duration of viraemias, a mid-point was used. It was assumed that an animal may have been viraemic for an additional 3 days before and after virus was actually detected. Therefore, for estimations of viraemia, the length of detected viraemia has been extended by 6 days beyond the period actually detected. For example, if virus was detected in one sampling only, a viraemia of 7 days was ascribed; if virus was detected in two samples taken a week apart, a viraemia of 13 days was ascribed. However, the actual duration of viraemia may possibly have been up to 6 days longer or conversely, up to 6 days shorter.

RESULTS

Identification of previously isolated viruses

Prior to 1994, eight strains of BLU virus had been isolated within China over a period of 15 years [1, 9], but these had not been identified to serotype. These isolates have now been identified as type 1 (Yunnan province, 1980, 1993; Xinjiang, 1989; Shanshi, 1993, 1994), and type 16 (Hubei, 1984; Sichuan, 1988; Shandong, 1989). An isolate from Inner Mongolia (1994) was tentatively identified as being related to type 17, but the status of this isolate is regarded as indeterminate at the present time [1].

New isolates from sentinel herds in Yunnan province

The herd at the highest altitude (Kunming) showed no bELISA seroconversions or virus isolations throughout the study. Seroconversions and virus isolations occurred at each of the other four sites (Table 1). In 1995, monitoring occurred at three sites, and a sole isolate (type 23) was made at Eshan. With the intensification of the sentinel herd programme in 1996 through the addition of herds at Jinghong and Dehong, seroconversions occurred in a further 27 animals, (Eshan 11, Jinghong 6, Shizong 4, and Dehong 6) and 90 isolates of BLU virus were made. (Some of the isolates from Jinghong and Dehong were made from young cattle that were already bELISA positive at the time of their initial sampling.) In 1997, there were a further 17 strains isolated from 10 animals. The total of 108 isolates consisted of types 1 (33 strains), 2 (3), 3 (4), 4 (17), 9 (1), 11 (1), 12 (11), 15 (17), 16 (19), 21 (1) and 23 (1).

The large majority of the isolates (104) came from cattle, rather than buffalo blood (4). Multiple infections often occurred, with the 108 isolates coming from 56 distinct infections of 35 animals. Eighteen (18) animals were infected with a single serotype, 14 were infected sequentially with 2 serotypes, 2 animals were infected with 3 serotypes and 1 animal with

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Table 1. *Bluetongue virus isolations, 1995/6/7*

	Serotype											
Location	1	2	3	4	9	11	12	15	16	21	23	Total
Dehong	1			**4**			**6**	**2**		1		14
Eshan	**18**, 5		**3**	**6**				**9**	**11**		*1*	53
Jinghong	**8**, 1	**3**		3	1	1	**2**, 3	**1**	**7**, 1			31
Shizong			**1**	**4**				**5**				10
All sites	33	3	4	17	1	1	11	17	19	1	1	108

Italics indicates the sole isolate made in 1995 (type 23); Bold indicates the types isolated in 1996; normal type indicates the types isolated in 1997.

Table 2. *Serotypes isolated at each sentinel site in 1996/7 and the time periods over which isolations were made*

		1996		1997	
Site	Serotype	First positive	Last positive	First positive	Last positive
Dehong	1			14 Aug	14 Aug
	4	17 Sep	3 Oct		
	12	14 Aug	9 Oct		
	15	7 Nov	7 Nov		
	21			11 Sep	11 Sep
Eshan	1	2 Jul	28 Aug	18 Sep	15 Oct
	3	9 Jul	28 Aug		
	4	4 Jun	28 Aug		
	15	17 Jul	28 Aug		
	16	25 Jun	21 Aug		
Jinghong	1	14 Aug	23 Oct	7 Oct	7 Oct
	2	21 Aug	4 Sep		
	4			31 Jul	7 Aug
	9			25 Sep	25 Sep
	11			3 Jul	3 Jul
	12	14 Aug	28 Aug	4 Sep	25 Sep
	15	31 Oct	31 Oct		
	16	26 Sep	31 Oct	27 Aug	27 Aug
Shizong	3	17 Jul	17 Jul		
	4	17 Jul	24 Jul		
	15	30 Jul	30 Jul		

4 serotypes (3 in 1996 and 1 in 1997). Most multiple infections occurred sequentially, but occasionally the same animal carried two serotypes within the same period of time. Not all serotypes were isolated from all locations, and the combination of serotypes present varied from site to site. Types 2, 9 and 11 were found only at Jinghong, type 21 only at Dehong, type 23 only at Eshan and type 12 at Jinghong and Dehong.

In addition to the 108 isolates, there were a further 25 samples which were positive by antigen capture ELISA, but from which no virus could be isolated. These are regarded as strains which were able to grow in ECEs but which were unable to adapt to cell culture [8].

Seasonality

Overall, viraemias in the sentinel animals were detected over a period of about 5 months in 1996, and about 3 months in 1997 (Table 2), transmission occurring in the warmer months of the year (June–November). Most isolates were obtained in 1996. In that year, the earliest isolate was obtained from the Eshan sentinel herd in early June, while the last was

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Table 3. *Duration of viraemias in sentinel cattle, 1995/6/7*

	Serotype										
Duration	1	2	3	4	9	11	12	15	16	21	23
6 days	*4		2	3	1	1	5	7	3	1	1
13	4			2			2		1		
20	1	1		2			1		2		
27	1								1		
34	2			1				1			
41	2			1				1			
48			1						1		
Mean (days)	19·5	20	20	17·7	6	6	9·5	15·0	18·3	6	6

* Figures refer to the number of animals with viraemia of the designated duration.

Table 4. *Comparison of bELISA and AGID results*

	AGID result				
	Negative	1+	2+	3+	Total
bELISA					
Negative	2281	58	1	1	2341
Inconclusive	223	23	0	3	249
Positive	518	635	82	51	1286
Total	3022	716	83	51	3876

obtained from Dehong in early November (serotype 15). The period of virus transmission was shortest at Shizong (viruses were only isolated in July). At the other three sites viruses were isolated over a period of 10–12 weeks. The months with the greatest apparent activity for virus transmission were July and August. There was no obvious pattern of sequential movement of the different serotypes between sites (other than possibly serotypes 3, 4, and 15 between Eshan and Shizong, which were relatively close and have the greatest similarities in topography).

In 1997, there were fewer isolates, but the general pattern was similar. At Jinghong, viraemias occurred from early July to early October. At Dehong and Eshan, transmission occurred over shorter periods in August and September/October respectively.

Duration of viraemias

The mean duration that was calculated for viraemias for each serotype fell within a range of 6–20 days, with overall mean for the 55 infections being 15·8 days (Table 3). There did not appear to be a great difference between serotypes. Seventy-eight percent (43/55) of viraemias were of not more than 20 days duration and 89% (49/55) were not more than 34 days long. Only 6 animals had longer viraemias (4 were estimated to be viraemic for 41 days and 2 for 48 days).

Serology

Comparison of bELISA and immunodiffusion methods for group antibody

The sensitivity of the two methods was compared using 3876 of the sera collected during the project (Table 4). Taking all degrees of AGID reactivity (1+, 2+, 3+) as positive, and excluding the inconclusive sera from bELISA positives, the latter test was still clearly more sensitive (33·2–22·0%) than the AGID in detecting BLU group antibodies.

Type-specific serology

Only a limited number of all available samples were tested by VNT, in part due to varying sample quality and quantity. Differing numbers of serum samples were tested against reference viruses of types 1–4, 7, 9, 12, 15, 16, 20, 21 and 23, with a total of 5400 tests completed. There was also limited testing for antibodies to type 17, mainly on sera from Inner Mongolia.

The cumulative results from these tests provided serological evidence for the presence of BLU types 1, 2, 4, 9, 12, 16 and 23 but no sera were positive for types 7 or 17. These serological results generally agreed well with the subsequent isolation of serotypes from the sentinel herds. However, it should be noted that type-specific serology was not completed on the sentinel cattle in 1996 and 1997 where efforts were largely directed towards virus isolation.

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DISCUSSION

Knowledge of the prevalence of BLU throughout the world and the distribution of serotypes is important from the viewpoints of both science and trade. This study represents the first detailed investigation of the epidemiology of the virus in China, albeit mostly in one province. As a first step, the use of sentinel herds of cattle was chosen as the most cost-effective way of gathering basic information, and, although this approach had not previously been utilized in China, the results have vindicated the methodology. The linkage of such field sampling techniques to the sensitive screening procedure provided by the antigen capture ELISA provided a rapid and relatively inexpensive way of maximizing the yield of BLU isolates.

More than 100 strains of the virus were recovered, falling into 11 serotypes (types 1–4, 9, 11, 12, 15, 16, 21 and 23). The serological tests confirm the general pattern of viruses isolated in the study. The discrepancy whereby there was a greater number of serotypes isolated than were detected by type-specific serology is explained by differences in the locations and years in which the sera were collected. Routine type-specific serology has not yet been performed on serum samples collected from sentinel animals in 1996 and 1997. The presence of these serotypes in China is consistent with the fact that all except type 11 have been isolated in one or more of the countries in south-east Asia, or northern Australia [6]. Serotype 11 has not been isolated in the region, Zimbabwe being the closest place of isolation [1]) but there is serological evidence for its presence in India [11]. It is of interest that no antibodies could be found to type 7, a serotype known to be present in Indonesia [12], and thought on serological grounds to be present in India [11]. However, further studies are needed to test, by type-specific serology, larger numbers of sera which have been collected from more provinces and over more years before conclusions can be drawn on the absence of particular serotypes in China.

The comparison between bELISA and AGID methods supported the widespread evidence from other countries [13], that the bELISA is a superior test to the AGID for the detection of group-reactive antibodies.

The fact that the isolates were made from cattle and buffalo emphasizes the important role which large ruminants play in the epidemiology of the virus. It is striking that such a large range of serotypes should be isolated within the one province over such a short time span. The spectrum of serotypes differed from site to site. This may indicate that the four locations involved had genuinely different viral populations, a reflection of their diverse surroundings and ecologies. The application of molecular genetic analyses to compare the topotypes of isolates of the same serotype between sites would be of considerable interest.

The study provides a considerable body of valuable data on the duration of virus persistence for 35 naturally infected animals, involving 11 serotypes. These data reinforce the viewpoint that naturally infected cattle have finite viraemias and are not persistently infected with BLU. In the present study, the longest viraemia was 48 days. While these data outline the duration of detectable viraemia, other studies have clearly shown the time in which a midge can become infected is probably much shorter [14]. It has been shown that the levels of virus in blood after 28 days are too low for infection of insect vector [14]. Collectively, this information has implications for quarantine holding periods for international movement of cattle, and indicates that, within the limitation of this study, seropositive cattle could be safely transported after a period of about 60 days in a virus- and vector-free area.

While it is known that types 1 and 16 can be significant pathogens in China [1], experimental studies of the pathogenic potential of these newly recognized serotypes for small ruminants are warranted. If any are found to be pathogenic, further serological surveys are justified to determine whether these viruses present a threat of disease due to their proximity to significant populations of sheep. With this knowledge, strategies for the development of new vaccines may be considered.

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