Table 8. Titration of bluetongue virus vaccine in the organs of chick embryos (0.2 mL in amnion).

Organs	Inoculum (vaccine)	Titration LC_{50}=Log
Heart	Y33-89F44, F46	$10^{3.33}$
Liver	F46, F48	$10^{3.5}$
Kidney	F52, F54	$10^{3.67}$
Brain	F42, F44	$10^{2.23}$
Amnion membrane	F53, F55	$10^{2.75}$
Amnion fluid	F56	no regular deaths seen

Table 9. Recovery of virulence of ECE-attenuated vaccine in sheep (Sichuan strain).

Date	Strain	Sheep without clinical signs		Clinical signs
		Leucocyte decrease	Fluctuation in body temperature	
26 Apr 1989	SWF22	2/2	1/2	0/2
3 Jun 1989	SWF30	8/10	3/10	0/10
Dec 1989	SWF35	1/3	1/3	0/3
4 Jan 1989	SWF30	0/10	0/10	0/10

Table 10. Virulence tests of ECE-attenuated vaccine in sheep (Yunnan strain).

No. of passages in sheep	Sheep no.	Blood inoculum 7 days post-infection	Clinical signs	Recovery of virulence
1	564	Y33-89F51	–	0/2
	565	from ECE 4 mL	–	
	576	Y33-89F113	–	0/2
	577	from ECE 4 mL	–	
2	578	from blood of	–	0/2
	588	564 and 565	–	
	581	from blood of	–	0/2
	582	576 and 577	–	
3	583	from blood of	–	0/2
	584	578 and 588	–	
	585	from blood of	–	0/2
	586	581 and 582	–	
4	587	from blood of	–	0/2
	588	583 and 584	–	
	589	from blood of	–	0/2
	590	585 and 586	–	

The successful protocol using ECE-attenuated isolates to produce vaccine at 33.5–35°C had been adapted from those of Mason and Neitz (1940) and Alexander et al. (1947). Immunofluorescence testing, a reliable application for testing vaccine virulence, showed that the vaccine titre was highest ($10^{3.6}$) at passage 47 (Table 12; Zhang Nianzu et al. 1991), and tended to decrease after F60. The results suggested that the optimum passage level for developing vaccine was F46–F60. There was no difference in the effectiveness of the venous and amniotic routes for virus passage.

Table 11. Protection tests with ECE-attenuated vaccine in Yunnan.

Trial	Inoculum	No. of sheep	Vaccine dose	Challenge days post-infection	Subclinical	Clinical	Protected Total
1	Y33-89F46	2	5 mL	35	–	–	2/2
	Y33-89F75	3	5 mL	35	+–	–	2/3
	control	2		35 (then observed for 39 days)	++	++	0/2
2	Y33-89F47	3	5 mL	35	–	–	3/3
	Y33-89F108	3	5 mL	35	–	–	3/3
	control	3		35 (then observed for 35 days)	++–	++–	*1/3*
Vaccinates							10/11
Control							*1/5*
	Y33-89F47	6	5 mL	14	1	0	5/6
	Y33-89F51	12	5 mL	21	1	0	11/12
	Y33-89F60	9	5 mL	21**	1	1	8/9
	Y33-89F113	2	5 mL	21**	0	0	2/2
Vaccinates							26/29
Total controls							*2/12*
Total							36/40

Table 12. Protection test of ECE-attenuated vaccine in Sichuan.

Date	Inoculum titre	No. of sheep	Vaccine and dose	Challenge inoculum	Challenge days post-infection	Protected/total
10 October 1981	10^{-1}	3	Y33-89F47,	Y33F16 459,	14	3/3
	10^{-2}	3	5 mL per dilution	= 50 ID_{50}		2/3
	10^{-3}	3				2/3
	10^{-4}	3				0/3
PD_{50}		$10^{-4.3}$/5 mL		= $10^{-3.6}$/mL		
26 September 1982	$10^{-0.93}$	3	Y33-89F51, 1 mL		21	3/3
	$10^{-1.69}$	3	per dilution	Y33F3 565,		3/3
	10^{-2}	3		= 0ID_{50}		3/3
	$10^{-2.39}$	3				2/3
	$10^{-2.69}$	3				0/3
PD_{50}				= $10^{-2.64}$/mL		
16 November 1985	$10^{-1.69}$	5	G56 +68,		21	3/3
			1 mL per dilution			
	10^{-2}	5		Y33F3 778,		3/5
				= 25ID_{50}		
	$10^{-2.38}$	5				2/5
	$10^{-2.6}$	5				1/5
	$10^{-2.9}$	5				1/5
PD_{50}				= $10^{-2.49}$/mL		

A comparison of vaccines in Yunnan and Sichuan showed slight differences between the two. First they differed in virulence. The products of the Yunnan virus failed to infect sheep only after passage 47 via ECE, in contrast to Sichuan's failure after passage 22 (Tables 9 and 10). While this suggested that the virulence of isolates from Yunnan was greater than that of isolates from Sichuan, the results of sheep cross-protection tests showed only slight cross-protection (Table 12). Mild clinical signs following inoculation with Sichuan inoculum were observed in sheep vaccinated with the Yunnan vaccine. The two local isolates were therefore classified as two serotypes. Retrospectively, Yunnan isolate was found (in 1994) to be BLU1 and the Sichuan isolate BLU16 (Zhang Nianzu these Proceedings).

In conclusion, ECE-attenuated vaccines have been developed successfully in Yunnan and Sichuan. For vaccine production, the recommended passage number via ECE was 46 to 60. The vaccination experiment showed that both vaccines gave a high protection rate with some cross-protection between the two. This procedure for ECE-attenuated vaccine for bluetongue is strongly recommended as the established standard technique.

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Cross-protection Studies of Bluetongue Viruses in Sheep

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Abstract

Fifteen sheep were divided into five groups and inoculated with USA origin bluetongue virus (BLU) serotypes 2, 10, 11, 13 and 17 respectively (10 mL/sheep). After 42 days they were challenged with Y33F20 virulent strain. Survival rates were 0/3, 1/3, 0/3, 1/3 and 1/3 respectively. Another 15 sheep in five groups were inoculated with Y33F20 virulent Yunnan BLU (10 mL/sheep) and challenged at 42 days with USA origin BLU2, 10, 11, 13 and 17. Survival rates were 0/2, 0/3, 1/3, 0/3 and 1/3 respectively. A further group of 12 sheep were inoculated as follows: six with W53 infectious blood 5 mL/sheep; three with cell-adapted W53 10 mL/sheep; and three kept as controls. At 42 days, all were challenged with SF6 (Sichuan) and W53 (Hubei) strains at 1 mL/sheep. In each group 1/3 survived, and all controls were sick or died. These results indicate that Yunnan, Sichuan and Hubei strains are different serotypes and that the Yunnan strain is different from serotypes BLU2, 10, 11, 13 and 17 of USA origin.

CROSS-PROTECTION experiments were developed for studying different isolates of bluetongue virus (BLU). Neitz (1948) was the first to use the technique for bluetongue. Subsequently many scientists worked to develop multiple vaccines, identifying and studying the properties of different BLU isolates (Alexander and Haig 1951; McKercher et al. 1957; Luedke and Jochim 1968). More recently, cross-protection tests have been used to identify specific properties of local BLU isolates from Yunnan (Zhang Nianzu et al. 1989), Hubei (Li Zhihua et al. 1989) and Sichuan (Lin Lihui et al. 1989) in comparison with standard serotypes BLU2, 10, 13 and 17.

Materials and Methods

Chinese isolates

1. Y33F20 and Y33F22; isolated from blood of bluetongue-diseased sheep with fever, passaged through sheep 20 and 22 times respectively, then stored at 4°C.
2. W53; isolated from the blood of experimental sheep with fever by inoculating specimen from a sick sheep, stored at 4°C.
3. Y26; Yunnan isolate, passaged in BHK21 cell 26 times, harvested and stored at –80°C.
4. WF5; Hubei isolate, passaged in BHK21 cell 26 times, harvested and stored at –80°C.
5. SF2 and SF6; isolated from blood of sheep with fever, inoculated with a specimen from Sichuan, passaged in sheep twice (SF2) and six times (SF6) stored at 4°C.

Standard foreign strains

Five BLU serotypes from the National Virus Standards Institute, USA, passaged in BHK21 cells and stored at –80°C, were used as standards.

Experimental animals

Healthy Xinjiang Merino sheep, tested as BLU-free before the experiment, were used, with body temperatures and leucocyte counts recorded.

Treatments

1. Fifteen sheep were divided into five groups (three sheep per group), given an inoculum of 1 mL/

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sheep of each standard BLU serotype, and challenged with 1 mL/sheep of YF20 isolate.

2. Fifteen sheep were divided into five groups (three sheep per group), given an inoculum of 1 mL/sheep of YF20 isolate, and challenged with 1 mL/sheep of each standard BLU serotype.
3. Twelve sheep were divided into three groups: six sheep were inoculated with 5 mL/head of W53; three sheep with 10 mL/head of WF5; and three sheep given no treatment as a control. All were challenged with 0.2 mL/head of YF22 at 42 days post-infection.
4. Eight sheep were divided into three groups: three sheep were inoculated with 5 mL/head of Y26; three sheep with 2 mL/head of YF20; and two remained untreated as controls. The two treated groups were challenged with 1 mL/head of SF2 + SF6 or W53 respectively and the control group was inoculated with SF2 plus SF6.

Observations

Observations followed each treatment and trial for 15 successive days, measuring clinical signs twice a day and leucocytes every two days. An animal with clinical signs, a 1°C increase in body temperature or a one-third decrease in leucocyte count, was considered to have a positive reaction.

Table 1. Cross-protection tests of Yunnan isolate with five standard BLU strains passaged in BHK21 cells.

Trial no.	Group	Inoculum		No. protected/ total no.
		Inoculation	Challenge	
1 (28 November 1985)	1	BLU1	Y33F20	0/3
	2	BLU10		1/3
	3	BLU11		0/3
	4	BLU13		1/3
	5	BLU17		1/3
2	1	Y33F22	BLU10	0/3
	2		BLU2	0/2
	3		BLU17	1/3
	4		BLU11	1/3

Results and Discussion

The results of cross-protection tests comparing a Yunnan isolate with standard BLU serotypes, and with local Yunnan, Hubei and Sichuan isolates, showed there was no cross protection within any two isolates of paired groups (Tables 1 and 2). Yunnan isolate was therefore different from BLU2, 10, 11, 13 and 17. Agar gel immunodiffusion (AGID), immunofluorescent antibody (IFA) and nucleic acid polyacrylamide gel electrophoresis (PAGE) indicated that the bluetongue group specific properties of the local isolates, including Yunnan, Hubei and Sichuan, were the same as those of BLU2, 10, 11, 13 and 17 (Hu Yuling and Peng Kegao 1989). However, these local isolates may belong to different serotypes: this aspect needs further study.

Table 2. Cross-protection tests in sheep with Yunnan (Y), Hubei (W) and Sichuan (S) local isolates of bluetongue viruses.

Trial no.	Group	No. of sheep	Inoculum	Challenge	Protected/ total
3	1	6	W53	Y33F22	2/6
	2	3	WF4	Y33F22	1/3
	control	3	–		0/3
4 (10 June 1987)	1[a]	3	YF26	SF2-6	1/3
	2[b]	3	YF20	W53	0/3
	control	2	–	SF2-6	0/2

[a] Inoculated 18 February 1989
[b] Inoculated 11 January 1986

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Development of Inactivated Vaccines for Bluetongue in China

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Abstract

Bluetongue virus (BLU) strains Y863F6 and WF13, passaged in BHK21 cell culture, were inactivated by Cobalt gamma rays or hydroxylamine for vaccine development. The inactivated viruses were passaged three times in BHK21 cell culture to test inactivation efficiency and were inoculated into sheep for safety tests. As a potency test, the vaccinated sheep were challenged with 100 $TCID_{50}$ of the homologous virulent viruses in sheep blood after vaccination. The effect on the BLU RNAs was tested by polyacrylamide gel electrophoresis (PAGE). Bluetongue virus was not killed by irradiation with 2.5, 5 or 10 million gamma radiation (Grad). The Y863F6 virus irradiated with more than 2.5 million Grad showed no cytopathic effect (CPE) in BHK cells but the virus that received one million Grad showed CPE in the inactivation efficiency test. Sheep inoculated with viruses irradiated with 2.5 million Grad did not show any clinical reactions in the safety test. At 42 days post vaccination, the 2.5 million Grad group had good protection (10/12), the 5 million Grad group had less (2/5) and the 10 million Grad group none (0/5). Thus 2.5 million Grad is the recommended dose for an inactivated vaccine. Hydroxylamine is the best inactivator for BLU vaccine. The inactivated viruses were blind passaged in BHK21 cell culture three times. No CPE developed. The 17 inoculated sheep did not show any clinical reactions in the safety experiment. On the 42nd day after vaccination, 5/5 vaccinated sheep showed no clinical reaction but 2/2 controls did. At 205 days after vaccination 7/10 vaccinated sheep were still protected. However, 7/10 control sheep showed clinical reactions in the potency test. PAGE showed that the amine chemical can destroy BLU genome RNA. Vaccines made from BLU virus killed by the amine chemical have been used against bluetongue disease in epidemic areas in Hubei Province. Of the 7878 sheep vaccinated, none have shown subsequent clinical reactions. Bluetongue disease has been effectively controlled in these areas.

THE key element in preventing bluetongue was the development of vaccines. An attenuated vaccine has been widely used in many countries, including South Africa, USA (Stott et al. 1985) and China. However, such vaccines have disadvantages, including abortion in pregnant ewes; a long residual period; and genetic recombination in multiple vaccines. Attenuated vaccines may lead to the occurrence of bluetongue disease in non-epidemic areas by reversion to virulence (Foster et al. 1968). It was thus necessary to develop an inactivated vaccine to prevent bluetongue. This paper reports on further experimentation in the development of an inactivated vaccine, based on previous studies by Zhang Nianzu et al. (1991) and Stott et al. (1985).

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Materials and Methods

Isolates

The four isolates used as parent stock for developing inactivated vaccines were:

- Y863F6, a local Yunnan isolate, passaged three times in BHK21 cells;

- YF13, local Hubei isolates passaged three times in BHK21 cells;
- Y33F4, a local virulent Yunnan isolate, from bovine blood in an epidemic area; and
- YWF4(116), a local virulent Hubei isolate, from blood of sheep in a high prevalence area.

Animals

Experimental animals were healthy bluetongue-negative Xinjiang Merino sheep, clinically healthy and free of antibodies as assessed by AGID.

Inactivation procedures

Inactivation radiation doses of Cobalt 60 gamma rays at 1, 2.5, 5 and 10 million gamma radiation (Grad) were used, from a cobalt source located in Atomic Applied Technique Institute, Sichuan Province. The chemical used was hydroxylamine (manufactured by Sigma, Germany). BHK21 monolayers with 70% cytopathic effect (CPE) due to bluetongue viruses were inactivated with gamma rays in a gamma-ray treated inoculum.

For chemical inactivation, the BHK21 cells were harvested the same way, frozen and thawed alternately, then were lysed by ultrasonication. The culture was concentrated to 1/10 volume and inactivated with hydroxylamine (Zhang Nianzu et al. 1991).

As safety tests (all repeated three times), BHK21 monolayer cells were inoculated with each of the inactivated vaccines, blind passage three times and the CPE recorded daily. Uninoculated cells were used as controls.

Radiation-inactivated vaccine trial

Two sheep were vaccinated with 1 mL each of gamma-ray treated inoculum, with a second dose given a week later. At 42 days postvaccination, the sheep were challenged with virulent virus at a dose of $100ID_{50}$ per sheep. Three other sheep were inoculated with untreated inoculum, while another two were kept unvaccinated as controls. Clinical signs were recorded, body temperature measured twice daily and leucocyte numbers measured every two days. Animals showing a 1°C rise in body temperature, a one-third decrease in leucocyte numbers and clinical signs were considered unprotected.

Chemically-inactivated vaccine trial

Five sheep were vaccinated with a chemically inactivated inoculum, 12 were vaccinated for morphology studies, and four were inoculated with untreated inocula as controls. Challenge with virulent isolates was at 42 days postvaccination. Similar supporting observations were made.

Evaluation

The microneutralisation test (Hu Yuling 1991) was used to evaluate vaccine effectiveness in generating antibodies.

Results

Comparison of the inactivated vaccines showed that there was no CPE in the test BHK21 cell cultures except for the group which received 1 million Grad (Table 1). This dose level was not used in the vaccination test in the two repeat trials. A few animals showed bluetongue signs and leucocyte changes in the repeat experiment at doses of 2.5, 5 and 10 million Grad (Table 2). The same results were obtained from all three replicate trials.

The vaccination tests showed that there were obvious differences of protection rate and level of neutralisation antibodies (Table 3) between or among each dose of gamma-ray treatment and treatments of inactivation (Table 4).

Table 1. Cytopathic effect (CPE) in BHK21 cell cultures inoculated with radiation- or chemically-inactivated bluetongue vaccine.

Treatment		BHK21 cell CPE (passage)			Remarks
		1	2	3	
Chemical	hydroxylamine	–	–	–	same in three trials
	control	++	+++	+++	
Gamma radiation	1 million	+	++	+++	
	2.5 million	–	–	–	same in three trials
	5 million	–	–	–	
	10 million	–	–	–	
	control	++	+++	++	

Table 2. Reactions of sheep after vaccination with gamma-ray inactivated bluetongue vaccine.

Vaccination	Gamma-radiation (MGrad)	No. of animals	No. of sheep with reactions			No. reacted/ total no.
			BLU	Leucocyte change	Clinical signs	
1st	2.5	2	0	0	0	0/2
	5	2	0	0	0	0/2
	10	2	0	0	0	0/2
	control	3	2	3	3	3/3
2nd	2.5	10	4	2	0	2/10
	5	3	2	2	0	2/3
	10	3	0	0	0	0/3

Table 3. Neutralisation tests of sheep vaccinated with gamma-ray inactivated bluetongue vaccine.

Group	Sheep no.	Gamma-radiation (MGrad)	Serum dilution 14 days post-infection					Serum dilution 42 days post-infection				
			1:2	1:4	1:8	1:16	1:32	1:2	1:4	1:8	1:16	1:32
1	871	10	+	–	+	–	–	+	+	–	–	–
	872		+	+	+	–	–	+	+	+	–	–
2	873	5	+	–	–	–	–	+	+	–	–	–
	874		+	+	–	–	–	+	–	–	–	–
3	875	2.5	+	–	–	+	–	+	–	+	–	–
	876		+	+	+	+	–	+	+	+	+	–
4	8811	control	0*	0	0	0	0	+	+	–	–	–
	882		0	0	0	0	0	+	+	–	–	–

* 0 indicates case in which animal was not inoculated with virulent inoculum but titration of sera was above 1:16.

Table 4. Protection rate of gamma-ray inactivated bluetongue vaccine

Challenge	Gamma-radiation (MGrad)	No. of sheep in group	No. of sheep that reacted			No. protected/ total no.
			BLU	Leucocyte change	Clinical signs	
1st	2.5	2	0	0	0	2/2
	5	2	2	2	0	0/2
	10	2	2	2	0	0/2
	inactivated vaccination	3	0	0	0	3/3
	control	2	2	2	2	0/2
2nd	2.5	10	2	2	0	8/10
	5	3	1	1	0	2/3
	10	3	3	3	0	0/3
	control	2	2	2	2	0/2

The protection test using chemically-inactivated vaccine showed there were no bluetongue signs and leucocyte changes in the five experimental sheep. (Morphological results are reported in another paper in these Proceedings.)

Discussion

The five sheep vaccinated with inactivated material were protected effectively (protection rate 100% 42 days post-vaccination) by the vaccine. These results

show that chemical inactivation is a valuable approach to developing a bluetongue vaccine.

The effectiveness of a radiation inactivation vaccine must focus on the dose of radiation. Failure to inactivate BLU occurred at 1 million Grad (Table 1) and failure to protect sheep from inoculation of virulent BLU at 10 million Grad (Tables 2, 4). This result differs from that of Campbell (1985) who reported that a dose of 6–10 million Grad had no effect on the vaccine's protective effect. The moderate dose of radiation in this experiment, 2.5 million Grad Cobalt-60, had a high protection rate (85% at 42 days post-vaccination), without any clinical and subclinical reactions after vaccination (Table 2). Possibly this was related to a lymphocyte transformation (Stott et al. 1979, Jeggo and Wardley 1982). This was similar to the results of Huismans animal vaccinated with purified VP2, which demonstrated that the 2.5 million Grad was not harmful to the VP2 protein The vaccine nucleic acid was not structurally changed by the inactivation procedure (Peng Kegao et al. these Proceedings).

In summary, hydroxylamine is a safe and effective inactivant for bluetongue vaccine. Gamma irradiation (2.5 million) by Cobalt-60 was recommended as an alternative method for inactivation.

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Analysis of RNA of Inactivated Bluetongue Vaccine by PAGE

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Abstract

Hydroxylamine and gamma-ray inactivated vaccines show a promising future. We have previously reported that, at a certain dose, gamma-rays could destroy the viral genome while maintaining immunogenicity; and that hydroxylamine could destroy pyrimidine while also maintaining immunogenicity. We now report having used RNA-polyacrylamide gel electrophoresis (PAGE) to analyse the vaccines to understand the mechanism of inactivation. The results uniformly revealed that viral segments 1 to 3 were degraded into low molecular weight compound after exposure to 2.5×10^6 rad on PAGE: segment 4 was partly degraded and segments 5 to 10 were affected. The hydroxylamine-inactivated samples showed slower migration and rocket-like bands. All controls displayed 10 bands with a typical profile of 3, 3, 3, 1. All inactivated samples failed to produce cytopathic effect (CPE) on BHK21 cells. Gamma-rays therefore inactivate bluetongue viruses by degrading their RNA. Hydroxylamine did degrade dsRNA but might affect the end of dsRNA or the pyrimidine. Whether the affected dsRNA could continue to serve as a template remains to be tested. RNA-PAGE could be used as a method to understand the mechanism of inactivation of bluetongue viruses.

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The Prevention and Control of Bluetongue Disease in China

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Abstract

This paper reports on the isolation of bluetongue virus, and the distribution, prevention and control of bluetongue disease in China since 1979. At present, the integrated preventative and control measures involve: control of infectious agents; checking of cattle and sheep imported from infected areas; monitoring and control of animals in genetically important stud stock; control of vectors; and developing vaccines with the major virus serotypes. Vaccination has already controlled the incidence of clinical disease in sheep in China.

BLUETONGUE disease is a non-contagious infectious disease that mainly affects sheep and other ruminants. The first diagnosis of bluetongue in China was made in 1979, and was followed by epidemiological and serological surveys throughout China. At that time prevention and vaccination were emphasised as control measures. This paper reports the surveys and control measures for bluetongue in China since 1979.

General Survey

An epidemiological survey of bluetongue was begun after the first outbreak in Yunnan in 1979 (Zhang Nianzu et al. 1989b): serological statistics from epidemic areas are shown in Table 1. The morbidity caused by bluetongue in epidemic areas was 8.2–35%, with mortality rates from 20% to 30% among the sick animals. The geographical distribution of bluetongue extended from the southern areas to as far north as 35–37°N. At the northern area's southern limit, at about 35–45°N, the incidence of the disease was low. Susceptible animals included sheep which had clinical signs and cattle which did not show clinical signs. Most affected were pure breed Merino sheep introduced from southern areas both domestic and abroad.

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Susceptible ruminants, including sheep in epidemic areas (Table 1) and cattle in 29 Provinces (Table 2) were surveyed using an agar gel immunodiffusion (AGID) test (Zhang Nianzu et al. 1989a). High positive rates for cattle occurred in the southern areas, while few positive cattle were found in Xinjiang and Inner Mongolia. The provinces of Qinghai, Heilongjiang and Jilin were bluetongue-free. The high prevalence of antibodies in cattle suggested they were the major potential source of epidemics in China.

Table 1. Serological survey using AGID of bluetongue in sheep and goats in epidemic Provinces, 1979–1989.

Year	Province	No. of animals surveyed	No. of positives (%)
1979–1984	Hubei	1972	146 (7.4)
1983–1989	Yunnan	19 290	3354 (17.4)
1984–1989	Hubei	3990	182 (4.6)
1986–1988	Inner Mongolia, Sichuan, Tibet	6342	1171 (18.5)
1987–1989	Anhui, Hubei, Hebei	8720	2553 (29.3)
Total		40 314	7406 (18.4)

Table 2. Serological survey by AGID of antibodies to bluetongue in cattle in epidemic Provinces, 1979–1989.

Year	Province	No. of cattle reared	No. positive	% positive
1979	Yunnan, Hubei		430	
1983	Yunnan	6 707 100	199	0.0029
1985	Yunnan, Hubei, Guangxi	15 586 900	4418	0.0266
1987	Yunnan, Shandong, Anhui, Hebei	17 173 700	381	0.0022
1988	Yunnan, Shandong, Hebei, Zhejiang, Xinjiang, Hainan	2 339 743	1 460	0.0624
1989	Total 26 Provinces (excluding Qinghai, Heilongjiang and Jilin)	98 824 909	12126	0.0123
Total	China	141 632 352	19014	0.0134

Control Strategy for Bluetongue in China

Strategies for controlling bluetongue in China have involved the control of acute outbreaks, monitoring, vaccination, quarantine, the control of products and the destruction of insect vectors.

The initial, basic but effective, strategy used to control the spread of the infectious disease was the killing of animals which were clinically ill during outbreaks and isolating the outbreak areas. During the acute outbreaks in China between 1981 and 1984, 4567 ruminants were killed. To monitor the prevalence of bluetongue, another strategy was the establishment of several sentinel flocks of animals in epidemic areas (described elsewhere in these Proceedings). These sentinels showed a decrease in the AGID positive rate from 36.6% to 2.3%.

Other strategies involved strict quarantine and testing. The introduction of breeds from both domestic and foreign regions with a history of epidemic bluetongue was controlled, with the AGID test used to identify infected animals. Germplasm, such as frozen semen, and the associated instruments for the artificial insemination of breeding animals, was also tested for the presence of bluetongue viruses.

On many farms, effective use of insecticides as animal dips decreased the attack of vector insects such as *Culicoides*.

Finally, the most important strategy for controlling bluetongue was vaccination, using attenuated and inactivated vaccines. These vaccines were developed successfully in China, a great research achievement. The results of vaccination were very successful in China, and this strategy will be emphasised and improved in future research.

The effectiveness of all these strategies for controlling bluetongue in China has been proved by the decrease in the incidence and serological prevalence of the disease in sheep and cattle.

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Developments and Advances in the Prevention of Bluetongue Disease

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Abstract

This paper reports developments and advances in the prevention and control of bluetongue disease in China. Since bluetongue was first recognised in China in 1979, there have been systematic studies of its pathogenesis, epidemiology, pathological processes, diagnosis, vaccination, disease monitoring and immune mechanisms. Two serotypes have been used in a modified live virus vaccine, and killed vaccine has been used to vaccinate sheep in isolated foci in southern China. Carried out one to two months before expected outbreaks, this vaccination program has controlled the incidence of clinical disease. A virus isolate from Northern China did not produce clinical disease when inoculated into sheep. The virus-carrying animals (cattle, goats etc.) are latent carriers and thus the source of viruses which cause disease. Development of more rapid diagnostic methods, applications of molecular techniques, monitoring of major serotypes and isolation of viruses should continue. Quarantine measures, disposal of diseased animals and annual or regular vaccination will assist in the control of disease and in minimising economic losses.

BLUETONGUE viruses (BLU) mainly attack ruminants. Sheep, particularly fine wool sheep (Merino), are most sensitive. BLU viruses cause bluetongue disease and produce high mortality. Cattle and other ruminants are often silent BLU carriers with no clinical signs, but are potential sources for the spread of disease. The importance of prevention is becoming more and more obvious in animal production.

Through many studies on bluetongue, much progress has been made in understanding BLU pathogenesis, epidemiology, immunology and appropriate research methodologies. However, as the viruses are carried by insects, the size of the epidemic area increases annually. The study of this aspect has attracted many scientists throughout the world.

In China, there were no records of this disease until the outbreak and isolation of bluetongue in Shizong, Yunnan in 1979. Many basic bluetongue studies, including aetiology, pathogenesis, immune mechanisms, clinical diagnosis, immunoserology and vaccine production, have supported the need to prevent the disease. This paper reviews the progress of preventative technology and its applications to bluetongue disease in China.

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Distribution of Bluetongue in China

Outbreaks of bluetongue in China have been reported in Yunnan (1979), Hubei (1983), Anhui (1985), Sichuan (1988) and Shanxi (1993). The geographical limits of the epidemic areas are confined to the southern part of China as far north as latitude 35–37°N. Serological surveys have shown that 29 Provinces have seropositive animals. The higher positive rates were in sheep in Yunnan and Guangxi among the southern provinces, and in goats in Inner Mongolia. In the other Provinces with seropositive animals, the positive rate was from zero to 4.7%. In cattle, the prevalence of bluetongue antibodies, using AGID, was 0.01%, except for Inner Mongolia and Qinghai, Jilin and Heilongjiang Provinces in the far north. No clinical signs have been found in any seropositive cattle or buffalo. No camels tested have been positive for antibodies.

Practical Techniques for Preventing Bluetongue

After the confirmation and isolation of BLU in 1979, a systematic study of ways of preventing bluetongue was conducted, with the Yunnan group as leaders collaborating with the National Quarantine Institute, Harbin Veterinary Research Institute (which belongs to the Chinese Agricultural Academy of Science) and the corresponding provinces. Bluetongue viruses were successfully isolated from sheep in Yunnan, Hubei, Anhui, Sichuan and Shanxi Provinces, and from sentinel animals in Inner Mongolia and Xinjiang, Shandong and Gansu Provinces. Inoculation experiments showed that none of the isolated viruses, whether from natural sick animals or from sentinels, could cause clinical signs in animals.

The major serotypes in China are now recognised as BLU1 and BLU16. Successful testing technologies have been evolved (AGID, cELISA etc.), and these have been standardised and expanded all over China. The successful production of egg embryo vaccines (from the Yunnan strain) and BLU16 (from the Sichuan strain) was an important fundamental step towards the prevention of bluetongue in China.

The comprehensive study to prevent bluetongue in China involved a primary stage and a vaccination stage.

Primary stage

Preventing and controlling the spread of bluetongue from the outbreak site: by using quarantine measures, isolation of sick animals, quarantine of the epidemic area, insect destruction on grassland where the epidemic occurred and surrounding areas and by slaughtering the sick and suspect animals. This stage lasted from 1979 to 1984. A total of 4567 animals (740 sick or suspected cattle and 3827 sheep) were slaughtered, which resulted in a sharp drop in the rate of seropositives in sheep from 35.9% to 2.3% compared to a seropositive rate of 16.3% in cattle and 8.9% in buffalo. It thus appeared that this approach was effective in preventing bluetongue in sheep.

Vaccination stage

The egg embryo attenuated vaccines of BLU1 and BLU16 and the inactivated antigen of these viruses were successfully produced. Many basic studies have been completed in the last decade. Preventing bluetongue in China was being accomplished through a comprehensive program of vaccination combined with the above sanitary measures. Progress in practical prevention included establishing sentinel herds, restricting the movement of and quarantining seropositive animals, and improving sanitation in animal housing and on farms. A total of 120000 animals were vaccinated (Table 1). The results showed that the protection rate of embryo attenuated vaccine was greater than 90%, with a period of immunity lasting one year, and up to 80% protection rate after one year. The inactivated vaccine gave immunity lasting more than six months. Full protection was gained by vaccinating two months before the epidemic season.

To establish vaccination schemes in areas where the BLU serotype infecting animals has not yet been identified, a combined vaccine (two types of attenuated vaccine) should be used to prevent multiple infections with bluetongue viruses. Comprehensive prevention procedures in China over the last decade have been fully successful in some previous outbreak areas in the southern Provinces. Further preventative strategies should focus on studies of vaccine development, vaccination, establishment of a standard vaccination regime, monitoring for BLU and antibodies in sentinel herds and strict protection of non-epidemic areas.

Table 1. Preventative vaccination of bluetongue in some Provinces of China.

Region	Years	Vaccine used	Number of vaccinated animals	Evaluation of outcome
Hongchiba, Sichuan	1989–1995	BLU1, BLU16 (chick embryo attenuated)	84000	Prevented bluetongue effectively
Xianfan, Hubei	1989–1994	BLU16 (inactivated antigen)	12000	Prevented bluetongue effectively plus protection rate of 70% 205 days post-vaccination
Jiangsu Province	1994–1995	BLU1, BLU16 (chick embryo attenuated)	16000	Prevented bluetongue effectively
Shanxi Province	1995	BLU1, BLU16 (chick embryo attenuated)	8000	Prevented bluetongue effectively

Open Forum and Finale

Introduction

RESTRICTIONS on the international movement of ruminants from bluetongue endemic areas to bluetongue-free areas, and even between countries with endemic bluetongue, have evolved from the proposition that once a cow, sheep, goat, buffalo or other ruminant has been infected with a bluetongue virus it may be infected for life. Thus both the live animal and its germplasm were considered risks by being potential sources of virus.

The information presented in these Proceedings, and especially in the next three papers, was discussed in various ways during the course of the Symposium. In the Open Forum, in the final stages of the Symposium, the following points were not disputed.

- Infection with a bluetongue virus is of finite duration. Virus components become undetectable, even by PCR, at the end of the life span of a red cell, approximately 150 days.
- The blood of infected ruminants is infective experimentally to other ruminants for a very much shorter period than this, certainly not longer than two months (the limit at which live virus has been isolated from blood): usually the period of viraemia in natural infections is much shorter.
- The excretion of bluetongue virus in semen can occur, but uncommonly, when a bull is infected with attenuated virus, and very rarely occurs in naturally infected bulls. Any excretion of virus into semen occurs only during viraemia.
- There is no evidence that immune tolerance is induced in cattle or sheep infected in utero.
- Both field and laboratory evidence suggest that bluetongue virus does not overwinter in cattle.

The value of diagnostic tests, and their interpretation, was also discussed in the Open Forum.

- The bluetongue complement fixation test is not routinely used in laboratories in the Southeast Asia and Pacific region, as represented by delegates at this Symposium, except for purposes of import or export.
- There was agreement that the complement fixation test is prone to error; is highly cross-reactive, causing many false positives; and is impossible to standardise internationally. Its use should be ended.
- Competitive enzyme linked immunosorbent assay (cELISA) was considered more sensitive and specific to the bluetongue serogroup than agar gel immunodiffusion (AGID), and should replace the latter where possible.
- The detection of bluetongue antibodies in serum collected from a ruminant should not be interpreted as meaning that the animal has live virus circulating in its body. The time since the animal may have been exposed to infection should also be considered.

Bluetongue Virus Infection of Postnatal Cattle

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Abstract

Bluetongue virus (BLU) infection of cattle is common throughout tropical and temperate areas of the world. Cattle appear to act as reservoir hosts in which virus amplification occurs. Haematophagous insects then transfer BLU to sheep and other ruminants which are more susceptible to bluetongue disease. Chronically infected cattle have been considered responsible for disseminating BLU, but this notion has not been adequately substantiated. Present knowledge suggests that vertical transmission is unimportant in BLU epidemiology. However, evidence confirms that typically viraemia is prolonged in BLU-infected cattle, but that the vast majority of infections are asymptomatic. Recent studies have characterised better the pathogenesis of BLU infections in cattle. The virus replicates primarily in the lungs and lymphoid tissues (lymph nodes and spleen) of infected cattle, and the association of virus with blood cells facilitates a prolonged viraemia of up to 50 days or even longer. Using polymerase chain reaction (PCR) tests, viral nucleic acid may be detected in blood cells for longer still (approximately 150 days), but there is no evidence that infectious virus can be recovered ('rescued') by insect vectors from cattle with blood that is positive by PCR analysis but not by virus isolation. We suggest that red blood cells facilitate prolonged viraemia in infected cattle, by protecting circulating virus from the immune system, and infection of haematophagous insects, which disseminate virus to other species. Furthermore, we conclude that viral nucleic acid persists in infected ruminants only for the life span of red blood cells (approximately 150 days in cattle and slightly less in sheep), and that infectious virus is present in blood for an even shorter period.

BLUETONGUE is an insect-transmitted viral disease that occurs sporadically among populations of sheep and certain wild ruminant species (Spreull 1905; Ozawa 1984, Erasmus 1985). Bluetongue virus (BLU) infection of cattle is common throughout tropical, subtropical, and some temperate regions of the world, whereas bluetongue disease typically is encountered only in temperate regions, or when susceptible ruminants are introduced into bluetongue-endemic regions within the tropics and subtropics (Gibbs 1983, 1992; Ozawa 1984; Barratt-Boyes et al. 1995). The significance of bluetongue infection in cattle is that virus amplification occurs in infected cattle before virus is disseminated by haematophagous insects. Cattle have thus been considered as reservoir hosts that facilitate 'overwintering' of BLU at times when the vector population is low or absent (Du Toit 1962; Nevill 1971; MacLachlan 1994; Barratt-Boyes et al. 1995). Suggestions that cattle serve as BLU amplifiers and reservoirs reflect the knowledge that BLU infection of cattle is very common in endemic areas, that invariably such infections are asymptomatic (Osburn et al. 1981) and, most important, that viraemia may be prolonged in infected cattle (Du Toit 1962; Luedke et al. 1969; Luedke 1970; Nevill 1971; MacLachlan et al. 1987, 1990b; Barratt-Boyes and MacLachlan 1994). Luedke et al. (1977a, b, c, 1982) proposed that persistently infected cattle were important BLU reservoirs while Howell (1963) considered bluetongue to be an emerging disease being spread throughout the world by persistently infected cattle. As a direct consequence of this notion of cattle as reservoir BLU hosts, countries free of the virus have restricted or banned the importation of ruminants and/or their genetic products (semen/embryos) from countries in which infection is endemic (Gibbs 1983; Roberts et al. 1993; Barratt-Boyes and MacLachlan 1994). The principal justification for such restrictions is the fear that BLU might

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be spread by trade and animal movement between countries. However, current evidence suggests that this risk is minimal when appropriate precautions are taken. Furthermore, as members of the BLU group exist in distinct, relatively stable ecosystems in different regions of the world (Gould and Pritchard 1990; de Mattos et al. 1994), bluetongue cannot be considered an emerging disease recently spread by persistently infected cattle.

Pathogenesis of Bluetongue Infection of Cattle

Although, typically, BLU infection of neonatal and adult cattle is asymptomatic, infection is followed frequently by a prolonged cell-associated viraemia (Du Toit 1962; Owen et al. 1965; Jochim et al. 1974; MacLachlan et al. 1987, 1990a, 1994; Barratt-Boyes and MacLachlan 1994; Katz et al. 1994). Infected cattle mount a prompt and high-titred humoral immune response but virus and specific neutralising antibody often circulate together in blood for some weeks (Luedke 1970; MacLachlan et al. 1987; Richards et al. 1988; Barratt-Boyes and MacLachlan 1995). Immune precipitation and immunoblotting studies have failed to identify virus-protein specific humoral immune responses that coincide with virus clearance (MacLachlan et al. 1987; Richards et al. 1988). Similarly, interferon production and virus-specific cellular immune responses are temporally unrelated to virus clearance (MacLachlan and Thompson 1985; Ellis et al. 1990). It has been well established that BLU is intimately associated with blood cells throughout viraemia and, logically, it must be this cellular association, and not antigenic drift in the virus nor an inadequate host response, which prevents rapid virus clearance (Luedke 1970; Heidner et al. 1988; Whetter et al. 1989; Barratt-Boyes and MacLachlan 1994, 1995; Katz et al. 1994; MacLachlan et al. 1990a, 1994).

Although viraemia in infected cattle is prolonged up to 50 days or even longer the duration of viraemia is finite, such that truly persistent infections do not occur (MacLachlan et al. 1990b). Once virus has been cleared from the circulation, animals show solid resistance to reinfection with the homologous BLU serotype, although fully susceptible to infection with other serotypes. For instance, Owen et al. (1965) noted that South African cattle that were infected with up to three different BLU serotypes in one year could be infected in the subsequent year with up to three additional serotypes. Given the prolonged viraemia that often occurs in infected cattle, in South Africa Du Toit (1962) and Nevill (1971) hypothesised, very plausibly, that BLU might maintain itself in an area during the coldest times of the year by means of a low level cycle of infection of cattle and insects. However, clearly the viraemia is not of sufficient duration to facilitate maintenance of virus in the long-term absence of any vector. Thus there is still uncertainty about the source of the BLU strains that reappear annually in parts of the United States where vectors are not found for extended periods during the winter months. Long distance dissemination by infected vector insects is a logical possibility, but one must also consider the possibility that BLU is vertically transmitted in the insect vector, or that there is an unidentified non-ruminant reservoir host. For example, very recent studies have shown that dogs and various wild carnivore species are susceptible to BLU infection (Akita et al. 1994; Alexander et al. 1994; Wilber et al. 1994) but whether they play a role in the natural cycle of BLU infections is unknown.

While it is obvious from the preceding discussion that infection of blood cells facilitates prolonged viraemia in BLU-infected cattle, the pathogenesis of infection of blood cells is not yet understood fully. Initial BLU replication occurs in the regional lymph node draining the site of virus inoculation, and virus replication initiates a prompt humoral and cellular immune response within the infected node (Barratt-Boyes et al. 1995). After primary viraemia, BLU replication principally occurs in lymphoid tissues of both infected sheep and cattle (Pini 1976; MacLachlan et al. 1990a; Barratt-Boyes and MacLachlan 1994) from which virus is released into the blood, where it associates with circulating blood cells. Virus may be isolated from all blood cells, and even transiently from plasma in the initial stages of infection, whereas late in the course of viraemia BLU is associated consistently with red blood cells (Luedke 1970; Whetter et al. 1989; MacLachlan et al. 1990a; Barratt-Boyes and MacLachlan 1994). Alstad et al. (1977) suggested that BLU infection of ruminant blood cells occurred as a consequence of infection of haematopoietic stem cells in bone marrow, as was earlier proposed for the related Colorado Tick Fever virus (Emmons et al. 1972). More recent studies in calves, however, indicate that infection of blood cells was not a consequence of infection of haematopoietic precursors in bone marrow, because only low titres of virus were present transiently in bone marrow, and infected cells were not demonstrated in bone marrow by immunohistochemical staining for viral antigens (Barratt-Boyes and MacLachlan 1994). The kinetics of BLU infection of blood cells clearly are consistent with infection of circulating cells and not with infection of bone marrow stem cells (MacLachlan et al. 1990a; Barratt-Boyes and MacLachlan 1995). The pathogenesis of infection of circulating blood cells awaits full clarification.

Recently, we have developed an hypothesis for the pathogenesis of BLU infections in cattle (MacLachlan 1994; MacLachlan et al. 1994; Barratt-Boyes and MacLachlan 1995). In this scenario, red blood cells facilitate prolonged viraemia in infected cattle by pro-

tecting circulating virus from the immune system. The red blood cells also facilitate infection of blood-sucking insects which disseminate virus to other ruminant species. We have shown that virus first replicates in lymphoid tissues, probably in mononuclear phagocytic cells (MacLachlan et al. 1990a; Barratt-Boyes et al. 1992, 1995). Then virus is released into the blood stream, and any cell-free virus immediately adsorbs to circulating blood cells, regardless of type. Only a few virus particles, at most, would be expected to associate with a single cell. Virus then replicates in, and subsequently kills, permissive cells such as monocytes or proliferating lymphocytes. Most infected mononuclear cells would be killed. As cell-associated virus can be expected to circulate for the lifespan of the infected cell, so platelets and neutrophils, given their very brief life span measured in hours or days, would be infected only very transiently.

Logically, the critical cell type in maintaining viraemia would appear to be the red blood cell because, unable to support viral replication because of its lack of a nucleus and necessary cellular machinery, it would not be killed after infection. We have observed that BLU will bind to, and penetrate, bovine red blood cells after in vitro infection (Brewer and MacLachlan 1992, 1994), and a similar phenomenon probably occurs in infected sheep (Nunamaker et al. 1992). Infection of red blood cells, with internalisation of virus, would allow the virus to circulate in sites inaccessible to neutralising antibody but available to blood-sucking insects, which would digest the infected red blood cell and free any 'passenger' virus. The mononuclear phagocytic system would not be expected to be stimulated to remove such affected red blood cells because of the small numbers of associated virions, possibly in the form of infectious viral cores. However, any phagocytosis by tissue macrophages of infected red blood cells that did occur could result in BLU infection of macrophages, which then would contribute to virus production during the initial viraemia. One assumes that removal of infected red blood cells by mononuclear phagocytic cells in the later stages of viraemia does not result in a secondary round of virus replication because of host humoral and cellular immune responses to the virus.

The approximate 150- to 160-day life span of the red blood cell of adult cattle (Jain 1986) is consistent with our proposed pathogenesis for the prolonged viraemia that may occur in BLU-infected cattle, and is identical to the interval when BLU nucleic acid may be detected in blood by PCR analysis after either natural or experimental BLU infection of cattle (Luedke et al. 1977c; Katz et al. 1994). Thus, although virus replication occurs transiently in tissues of infected cattle, viraemia persists as long as blood cells that harbour the virus continue to circulate. Virus clearance occurs only when the last infected red blood cell is subjected to phagocytosis because of its senescence. There is no evidence for the longterm persistence of BLU after infection of cattle (MacLachlan et al. 1990a; Barratt-Boyes and MacLachlan 1995). In very recent studies, we have clearly shown that bovine blood containing viral nucleic acid (as determined by PCR analysis) but not infectious virus (as determined by isolation), is not infectious to sheep, nor to vector insects that ingest such blood or are intrathoracically inoculated with it (MacLachlan et al. 1994; Tabachnick et al. 1996). This suggests that cattle whose blood contains BLU nucleic acid, but not infectious virus, are unimportant in the natural cycle of BLU infection.

Congenital Bluetongue Infection

The significance of BLU infection of the bovine foetus has been a topic of much scientific disagreement, as reviewed by Osburn and MacLachlan (these Proceedings). All recent observations, including studies with the same viruses, have failed to confirm the findings of Luedke et al. (1977a, b, c, 1982) that bovine foetuses infected in early gestation are born persistently or latently infected with BLU. Invariably, both natural and experimental infections of bovine foetuses in early gestation with BLU, and with the closely related epizootic hemorrhagic disease virus (EHD), have led either to foetal death or to cerebral malformation in calves that survived in utero infection (Richards et al. 1971; Barnard and Pienaar 1976; MacLachlan and Osburn 1983; MacLachlan et al. 1985; Roeder et al. 1991). Furthermore, precolostral serum from calves infected early in gestation invariably contained antibodies to the virus with which they were infected. Foetuses infected later in gestation (after gestation day 150) do not develop severe teratogenic defects (Jochim et al. 1974). Finally, it must be stressed that most pregnant cattle naturally or experimentally infected with BLU produce normal, uninfected calves.

Scepticism regarding the role of chronically infected cattle in the epidemiology of BLU infection is encouraged further by observations of natural infections in ruminants. In an extensive study in the western United States, virus isolation was carried out on 8751 blood samples from cattle, with BLU being isolated from 206 samples. Isolation was highly seasonal: virus was never isolated from bloods collected from mid-December through June. These data suggest that it is the presence of suitable vectors that is critical to the survival of BLU in the western United States, rather than vertical transmission of virus from persistently infected cattle (Stott et al. 1985). Similarly, although BLU has caused disease epidemics on the Iberian Peninsula, in Greece, and in the Okanagan Valley of Canada, infection has not become endemic in those areas, as would be predicted if vertical trans-

mission and persistent infection of cattle did occur (Gibbs 1983, 1992; Barratt-Boyes and MacLachlan 1995). As these epidemiological studies have failed to identify chronically infected ruminants, it is obvious that BLU will not persist in an area in the absence of a suitable biological vector. Analysis of BLU isolates from different parts of the world indicates that viruses from each area have distinct topotypes (Gould and Pritchard 1990; de Mattos et al. 1994). This suggests that the viruses in each area are not recent introductions but have evolved in those regions over considerable periods of time. Thus all available evidence indicates that BLU has not been disseminated throughout the world by persistently infected cattle.

In summary, one can no longer accept the once popular contention that truly persistent, immunotolerant BLU infection of cattle could occur as a sequel to infection of the bovine foetus. Furthermore, it is very clear that ruminant germplasm from non-viraemic seropositive and seronegative animals can continue to be moved without risk from areas of the world in which BLU infection occurs to those in which it does not.

Conclusion

Although the concept of truly persistent infection of cattle has been rejected because of lack of convincing evidence, BLU infection of cattle is likely still to be very significant in the complex epidemiology of natural BLU infection. Infected cattle have prolonged viraemias, in the course of which the infected animals can act as virus-amplifiers and reservoirs from which virus may be transmitted by biting insects to other ruminants. Because the concept of persistent BLU infection in cattle has not been adequately substantiated, restrictions on the movement of ruminants and their germplasm should now be based on our understanding that the duration of viraemia is finite, reflecting the lifespan of red blood cells with which the virus associates, and that recovered animals show solid immunity to reinfection with the homologous serotype. On this basis there appears to be little justification for the trade restrictions that have, at times, been imposed on producers of ruminants and ruminant germplasm in areas of the world in which BLU infection occurs.

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Evidence Against Congenital Bluetongue Virus Infection of Cattle and Virus in Semen

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BLUETONGUE virus (BLU) infection of cattle is common throughout tropical and temperate areas of the world. Cattle are most commonly infected by the biting *Culicoides* midges. However, there have also been reports of vertical transmission to the developing foetal calf or, through virus infected semen, to susceptible cows. Congenital infections have led to speculation that infection of immunologically immature foetal calves may lead to immunological tolerance and persistent viraemias.

Immune tolerance and persistent infection

Experiments were designed to determine if immunological tolerance and persistent viraemias occur in cattle. Calves received intrafoetal inoculation of the US strains of BLU10 or BLU11 UC2 or UC8 (which were laboratory adapted) at either 120–125 or 243 days of gestation. The calves were observed 12 and 20 days following inoculation, and/or at birth, for signs of infection, for antibodies in precolostral serum and for physical deformities. Among the group inoculated at 120–125 days of gestation were calves which could not walk, with severe hydranencephaly and precolostral sera showing antibodies to BLU virus. No virus was recovered from the tissues or blood of any calf at birth. In the calves inoculated at 243 days of gestation, those infected with UC8 were delivered prematurely, and birth weights were low. The UC2-infected calves were born on the expected parturition dates and were normal in appearance. Brain lesions were minimal and consisted of glial nodules. All calves seroconverted, as precolostral antibodies were present. One of the calves that had been infected for 36 days had cleared virus. These studies showed that transplacental infection with laboratory-adapted bluetongue virus can cause infection of the foetal calf. Whether there are foetal malformations or whether normal calves are born depends on the stage of gestation at which infection occurs. All the foetal calves had seroconverted, indicating that neither immune tolerance nor viral persistence occurs in congenitally infected calves.

Virus in semen

Fifty seropositive bulls from artificial insemination units were studied to determine if they shed bluetongue virus in semen. No virus was recovered from the semen of any bulls. Studies on seropositive bulls indicate that virus shedding in semen is rare, and it did not occur in this group of bulls.

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Characteristics of Naturally-occurring Bluetongue Viral Infections of Cattle

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Abstract

For 15 years, herds of cattle have been used as sentinels to detect the presence of bluetongue virus (BLU) in the northern part of Australia's Northern Territory during the active season for the principal *Culicoides* vector species. Blood samples have been taken from 848 sentinel cows, steers and bulls weekly or twice weekly. The blood has been cultured for bluetongue and other viruses. Semen from exposed bulls has also been tested for virus throughout the monitoring period, and calves born from exposed cows have been examined pre-suckling by virus isolation and serological techniques. All eight BLU serotypes found in Australia have been represented in the sampling results, although there have been no predictable patterns in the serotypes which are active each year or in the number of sentinel cattle infected. Different BLU serotypes varied in the duration of the viraemia they caused, with most (99.5%) viraemic periods lasting from less than 2 weeks to 2 months. In these natural infections, possible BLU contamination of the semen was observed only once, and there was no evidence of BLU infection of 79 calves which were *in utero* when their mothers were naturally infected.

KNOWLEDGE of the characteristics of viral infections can come from observations of either natural or experimental infections. Data on natural infections are often imprecise: the time of initial infection is unknown, as may be the infection's duration and other aspects, depending on the observer's ability to access and monitor infected animals. Studies of the pathogenesis of viral infections, and the subsequent immune response, therefore frequently rely on experimental infections using viruses isolated in culture systems.

In the case of bluetongue virus (BLU), experience has shown that viruses passaged in cell cultures have markedly reduced pathogenicity in sheep (Gard 1987). Conversely, virus strains attenuated in cell culture have been found to cross the placenta of sheep and cause foetal malformations (Parsonson 1992). Thus information derived from experimental infections cannot be used as a guide to the characteristics of natural infections.

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Old Controversies Regarding Bluetongue Infections of Cattle, Now Mostly Resolved

As research in South Africa showed that cattle in bluetongue epidemic areas have a high prevalence of infection without showing disease (Du Toit 1962), cattle are considered asymptomatic reservoir hosts in that country (Erasmus 1975).

However, there has been considerable confusion over whether BLU causes disease in cattle. As shown in the review by Hourrigan and Klingsporn (1975), clinical signs have been reported in cattle from which BLU has been isolated, and abortions and foetal malformations have been attributed to these infections. However, subsequent analysis of such reports has suggested problems of misdiagnosis (Du Toit 1962; MacLachlan et al. 1992). Bluetongue infections are now no longer considered to cause overt clinical disease in cattle, although infection during early pregnancy may possibly, but rarely, cause reproductive wastage (MacLachlan et al. 1992).

Another issue addressed at length by researchers is whether bluetongue viruses are excreted in bovine semen. Such a possibility has implications for the reg-

ulation of trade in germplasm. Most concern has resulted from a report that one bull was persistently infected and shed virus for prolonged periods in its semen. This possibility has been substantially refuted (MacLachlan et al. 1992), and it is now considered that bluetongue viruses may be found in bull semen only rarely, and even then only during the viraemic period.

The Monitoring of Sentinel Animals

The sentinel herd system has been described elsewhere by Daniels et al. and Melville et al. in these Proceedings. At Coastal Plains Research Station (CPRS), near Darwin in Australia's Northern Territory, the experience developed over many years has shown that arboviral infections of livestock are common and that known BLU vectors are present all year round. Hence CPRS is an ideal site for monitoring the incidence of natural BLU infections, and also for conducting experimental work to determine the specific effects of these infections. Bluetongue vaccines have not been used in Australia, nor has their use been reported in neighbouring countries. Bluetongue isolates at CPRS should therefore be wild type viruses, without the incorporation of genetic material from vaccine strains.

In addition to the monitoring each year of the incidence of infections of various BLU serotypes in sentinel steers, observations have also been made on bulls and pregnant cows. Since 1984, bulls have been monitored for BLU presence in semen during episodes of natural infection. During the periods of maximum BLU activity January to May/June each year semen and venous blood samples have been collected and inoculated intravenously into indicator sheep (two per sample), and the sheep monitored serologically for evidence of infection. This system has been suggested as the preferred and most sensitive isolation system for BLU in semen (Parsonson et al. 1981).

Since 1984, cows have been mated during the season of maximum BLU activity and monitored by virus isolation weekly and pregnancy testing monthly. The cows have then been closely supervised at calving so as to allow collection of a colostrum-free serum sample from calves before the first suckling. This has provided data on the natural infections in cows during gestation, and on any reproductive failures or calf deformities, as well as serological data on any intra-uterine viral infections.

Incidence of Natural Infections

Gard and Melville (1992) presented data on the incidence of natural BLU infections in sentinel steers that occurred at CPRS from 1981 to 1990. This paper adds data for the period 1991 to 1995, and extends previous data by additional observations in other livestock monitored at CPRS during the relevant periods (Table 1).

Table 1. Natural bluetongue virus (BLU) infections of sentinel cattle at Coastal Plains Research Station, Northern Territory, Australia 1981–1995.

Year	No. of cattle monitored	No. of cattle infected BLU serotype							
		1	3	9	15	16	20	21	23
1981	86	1						3	
1982	70				1			7	5
1983	60	1						1	
1984	45	21			1			8	
1985	46			3					1
1986	46	17	4	1	1	8			
1987	46		22						
1988	46	21				39			
1989	52	2	34						36
1990	52	No bluetongue infections occured this year							
1991	51		6						
1992	65					47	2		
1993	65	26							
1994	60	40						6	
1995	58						41	54	

During the 15-year period, all eight of the BLU serotypes known in Australia (Gard and Melville 1992) were isolated from sentinel cattle at CPRS. The sentinel herds were subject to 31 cycles of BLU infections, during which 460 infections occurred among the 848 cattle being monitored. The most commonly observed BLU serotype was BLU1 (Table 1), which was present during eight of the 15 years. Infections with other serotypes occurred less frequently, in no discernible pattern.

BLU21 was present each year from 1981 to 1984, then not observed again until 1994. BLU9 and 20 infections were observed only twice, while BLU15, 16 and 23 have been observed only three times during the 15-year period. As they are detected irregularly, where are these viruses maintained in the intervening years?

In all 15 years except 1987, 1990, 1991 and 1993, more than one serotype infected the observed animals. In 1990, no BLU viruses were isolated at all, while in 1986 five different serotypes were encountered. In seven specific years, two serotypes were active, while in three years three serotypes were isolated.

The incidence of infection with each serotype varied markedly. During the 15 years, 31 cycles of infection occurred (as evidenced by the isolation of a serotype in any year). In eight of these infection cycles, only one isolate was made of the active serotype. However, these isolations occurred from 1981 to

1986, after which the isolation system was changed to include prepassaging of samples intravenously in embryonated hen eggs followed by passage in mosquito cell cultures (C6/36) before inoculation into BHK21 cultures. Use of this three-stage isolation system has greatly enhanced the efficiency of isolation (Gard et al. 1988). Nevertheless, even in recent years there has been great variability in the recovery rate of viruses. For example, in 1995 BLU21 was isolated from 54/58 cattle, while in 1992 BLU20 was isolated from only 2/65. Serological monitoring of exposures showed that only those two animals had been exposed to BLU20 that year. The isolation system in current use thus appears to be quite sensitive.

The overall picture that emerges is of eight BLU serotypes circulating unpredictably in the geographical location, sometimes in a large wave of infection involving most animals and sometimes infecting only a few animals. The data suggest a threshold of sensitivity for the detection of infections by sentinel animals, with sentinel group size as a factor in the detection of infections. When not being spread by waves of infection, a BLU serotype may perhaps be maintained in the environment by slow spread from one animal to another.

Duration of Viraemia in Natural Bluetongue Infections of Cattle

The period of detectable viraemia is important epidemiologically, as it is during this time that an infected animal is infective to vectors, and hence potentially active in supporting a cycle of infection. The movement of animals during the viraemic period may move virus to a new location, and may initiate a new cycle of infection there if competent vectors and susceptible mammalian hosts are present. Fortunately, the period of BLU viraemia is relatively short, as BLU does not establish persistent infections (MacLachlan et al. 1992).

Viraemia has been observed in 31 cycles of natural BLU infections involving eight serotypes over a period of 15 years at CPRS (Table 2). As the data are based on weekly virus isolations, an isolate recovered from an animal at only one sampling has been considered to indicate viraemia of less than 2 weeks duration.

Some serotypes have been associated with quite short viraemic periods while others have shown longer durations, with most infected animals having had detectable viraemias lasting less than 2 weeks (Table 2). BLU15 and 9 were isolated for only one and two weeks respectively, while BLU1 and 16 often caused viraemia lasting up to 8 weeks. The least frequently isolated serotypes (Table 1) had the shortest periods of viraemia.

Table 2. Duration of detectable viraemia in natural BLU infections in cattle, Coastal Plains Research Station, Northern Territory, Australia, 1981–1995.

BLU serotype	No. of cattle showing viraemia (weeks)								
	<2*	<3	<4	<5	<6	<7	<8	<10	<13
1	52	37	26	7	4	2	1		
3	22	17	16	5	5	1			
9	3	1							
15	3								
16	13	21	26	18	12	1	2		1
20	24	14	3	2					
21	23	21	27	8					
23	19	10	8	5					

*viraemia of less than two weeks duration.

Only one animal, a bull, had a period of viraemia lasting more than 10 weeks, although the data were equivocal. In most animals throughout the period of detected viraemia, virus was isolated at each sampling during the period, although occasionally not being detected at a weekly sampling. In the case of bull no. 23, with a BLU16 infection in 1988, virus was isolated on 29 February, 3 March and 7 March. There was a single isolation on 11 April and two further isolations on 28 April and 15 May. Hence in this bull, BLU16 was detected intermittently over a period of 67 days, during which time BLU1 was also isolated (on 18 and 21 April) from the animal.

Overall, the data confirm that persistent or prolonged viraemia is not a feature of natural BLU infections, and that the viruses are usually cleared from the circulation within periods ranging from less than two weeks to two months.

No Evidence for Congenital Bluetongue Infections of Calves of Sentinel Cows

Observations were made of pregnant cattle exposed to natural BLU infections (Table 3): data for the period 1984 to 1989 have been presented previously (Melville and Gard 1992). Available data includes the number of BLU infections in monitored cows; the number of calves born from such cattle from which a pre-suckling serum sample was obtained, and the number of calves from which a blood sample was tested by virus isolation (Table 3).

In some years, very few cows were infected and these did not become pregnant, so these years are not represented in the data. In other years, some cows were infected with two serotypes at different times. For these animals there were two periods during

which bluetongue viruses could have crossed the placenta. Table 3 therefore shows the number of BLU infections observed in cows rather than the number of cattle infected.

In this study of natural BLU infections, no calf of a cow infected during pregnancy was viraemic at birth, nor were homologous antibodies detected in any calf sampled before the ingestion of colostrum. Although abortions and dummy calves were observed in some years, there was no evidence that BLU infection was responsible in these cases (Melville and Gard 1992). There was also no evidence in these studies for transplacental BLU infection of the bovine foetus.

Some comparative published reports suggest that BLU infections in pregnant cows can cause abortions or birth of 'dummy' calves. These reports are based on several types of observations. In the most artificial experimental situation, direct inoculation of foetuses with viruses adapted to cell culture has resulted in cases of hydranencephaly, in both South Africa and North America (Barnard and Pienaar 1976; MacLachlan et al. 1985; Thomas et al. 1986). In other experiments, where pregnant cows rather than foetuses were inoculated with cell culture-adapted BLU, no foetal pathology or evidence of foetal infection was found (Parsonson et al. 1987; Roeder et al. 1991).

However, naturally-occurring intra-uterine BLU infection of hydranencephalic calves has been indicated in California by the detection of BLU antibodies in pre-suckling (pre-colostral) sera from two such cases (McKercher et al. 1970). No reports of equivalent precise observations on cases of teratogenicity in cattle in South Africa are known to the authors, but hydranencephaly in calves has been reported as a recognised problem (Zumpt et al. 1978). Serological data from affected calves pre-suckling were not reported, but serology in calves and dams indicated BLU and Akabane virus infections of the sampled animals. The clinical syndromes described did resemble those reported after BLU inoculation of bovine foetuses (Barnard and Pienaar 1976; MacLachlan et al. 1985).

Epidemiological observations by MacLachlan et al. (1985, 1992) have suggested that, under natural conditions, abortions and foetal malformations do not have an appreciably greater incidence where BLU infections are active. At CPRS such occurrences have not been attributed to BLU infections (Melville and Gard 1992).

However, the other evidence summarised above does suggest that hydranencephaly is a possible outcome of intra-uterine BLU infections. It would be useful to establish the conditions under which this could occur. In sheep, cell culture-adapted viruses, but not wild strains, have crossed the placenta and been associated with teratogenicity (Parsonson 1992). There arises the question whether cell culture-adapted viruses, such as vaccine strains, may have a similar effect in cattle and if such strains have been circulating in nature in North America (Osburn et al. these Proceedings) and South Africa, as a possible explanation of the BLU-associated cases of hydranencephaly reported in those countries. Such considerations relevant to cattle may have implications for other countries contemplating the use of live attenuated vaccines.

Non-excretion of Bluetongue Viruses in the Semen of Naturally-infected Bulls

From 1984 to 1995, bulls were studied each year at CPRS to detect any BLU in semen (Table 4), as well as to record viraemias for each of the eight serotypes circulating through the sentinel groups during that period (Table 1). Data for the period 1984 to 1988 have been presented previously (Gard et al. 1989).

Table 3. Observations on calves born of cows naturally infected with bluetongue virus (BLU) during gestation, Coastal Plains Research Station, Northern Territory, Australia, 1984–1994.

Year	No. of BLU infections in pregnant cows	No. of possible foetal exposures possibly producing antibodies in pre-suckling calves	No. of calves viraemic at birth/no. of naturally-infected cows
1984*	12	4	not tested
1988	23	10	0/9
1989	33	30	0/19
1991	2	1	0/1
1992	28	13	0/19
1993	12	5	0/8
1994	22	18	0/23

*Animals located at Berrimah Agricultural Research Centre, Darwin.

Table 4. Natural bluetongue virus (BLU) infections of bulls, Coastal Plains Research Station, Northern Territory, Australia, 1984–1995.

Year	No. of bulls	No. infected BLU serotype							
		1	3	9	15	16	20	21	23
1984	5	4						1	
1985	6								
1986	6								
1987	6		2						
1988	6	3				6			
1989	7		6						7
1990[a]	7								
1991	12		1						
1992	12					12	1		
1993	12	10							
1994	8	5							
1995	9						4	7	
Total		22	9			18	5	8	7

[a] No blue tongue infections occurred at this station (Table 1).

During these observations of natural infections, BLU was detected only once in the semen of a bull, and this occurred while the bull was viraemic, not at any other time. In 1984, Gard et al. (1989) observed a seroconversion to BLU21 in a sheep inoculated with semen from a bull that had also seroconverted to that virus. As seroconversion occurred in only one of two sheep inoculated with the semen, the authors were uncertain whether the observation was due to infected semen or due to insect transmission of virus. However, because the failure of sheep to respond to inocula of BLU21 in infected blood has been observed subsequently (Melville unpublished data), the observation of seroconversion in the sheep inoculated with potentially-infected semen should not be discounted.

Excretion of BLU in semen from naturally-infected bulls must be considered a rare event since only one possible transmission of infection was observed from 69 separate natural infections (Table 1). However, experimental studies (Melville and Kirkland 1995) have resulted in excretion of BLU in semen, detected by sheep inoculation as used in the present studies. Such experiments have shown that excretion of viruses is more likely in older rather than younger bulls, occurs only during the period of viraemia, and more frequently has involved cell cultured rather than wild virus (although in one experiment BLU23 unpassaged in cell culture was detected in semen from aged bulls). Detection of virus in semen is invariably associated with the presence of blood in the semen (Melville, pers. comm.). These factors should be remembered when interpreting older reports in the scientific literature.

Discussion

The monitoring of sentinel cattle for BLU infections over a 15-year period has provided an invaluable opportunity of observing the real events associated with natural infections, illustrating again the benefits of a well-designed, prospective approach rather than trying to interpret retrospective observations.

Thirty-one cycles of infection in the sentinel cattle herds resulted in 460 BLU infections among the cattle (Tables 1 and 3). Among these, 459 periods of viraemia were clearly of less than two months duration, while the one equivocal observation suggested a viraemia lasting less than three months. There was no evidence of infection in 79 calves born to cows infected with BLU during the gestation period.

The data from 460 infections of cattle, 79 calves from naturally-infected dams and examination of the semen of 62 infected bulls show that none of these natural infections resulted in persistent infections that could lead to persistent shedding of BLU in semen, or that could offer a means of maintaining BLU in the local environment. The changes in serotypes active from year to year (Table 1), and the irregularity with which they were manifest, also suggest that the bluetongue viruses that were isolated were not maintained in the district by persistently infected animals. The pattern was rather one of the local circulation of viruses introduced from other foci of infection.

These observations of natural infections add further evidence to refute earlier hypotheses of BLU epidemiology. There is no evidence that natural infections with wild BLU strains result in the clinical disease of cattle, nor that such infections are associated with infection of the foetus or reproductive wastage. There is evidence that excretion of wild BLU in the semen of infected bulls is a rare event, and associated only with the period of viraemia. All 460 infections, observed in a range of male and female stock of varying ages, were self-limiting.

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Diagnostic Techniques for Bluetongue Viruses

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The Biology of Bluetongue Virus Infections

Our current knowledge of livestock infections with bluetongue viruses has improved significantly with the introduction of modern technology, and with a better appreciation of issues that affect the reproducibility and quality control for diagnostic tests. In particular, we now have a more informed understanding of the interactions between bluetongue virus and different animal species; of animals' responses to infection; and of the persistence of virus in nature. These issues are extremely important for disease control and for the safe movement of animals between regions and countries.

The length of time during which a bluetongue virus is present in the blood, and during which antibodies are produced and persist, will vary, especially between cattle and sheep. In the early stages of bluetongue infection, up to the sixth or seventh day after an animal has been bitten by an insect, virus is only present in the bloodstream (ie. the animal has viraemia). The level of virus in the blood reaches a peak at about seven to ten days after infection and this may coincide with a fever. In some animals, antibodies to the virus first appear at about this time. However, the ability to detect antibodies in the early stages of infection depends on the type of test and the type of antigen to which the antibodies respond after the first week of infection. Viable virus and antibodies coexist in the bloodstream for a variable number of weeks, usually not more than about three to four weeks in sheep and four to eight weeks in cattle. Virus fragments, especially RNA, may be found in blood samples for much longer periods, as shown by polymerase chain reaction (PCR) testing. However, we do not believe these fragments are able to infect insects or animals. After the virus is completely gone, antibodies remain in the bloodstream, perhaps for a year and sometimes for life. The measured length of these antibody periods depend on the species of animal, the possibility of further infections with related viruses, and the type of test used.

Diagnostic tests can be directed towards the virus; towards group antibody (antibodies common to all bluetongue viruses); or towards serotype-specific antibody (antibodies directed against the antigens unique to viruses of a particular serotype).

Tests for Virus, Antigen and Nucleic Acid

Compared to tests for antibody, most tests to detect bluetongue virus, antigen or nucleic acid take longer to obtain results or are more expensive. However, these techniques may be the only option during the very early stages of infection.

Animal inoculation

Animal (especially sheep) inoculation has often been used as a standard for the detection of viable bluetongue virus and generally has high sensitivity. This approach, which depends on the availability of susceptible animals, is usually expensive and is not suitable for processing large numbers of specimens. The method allows the inoculation of a large volume of specimen. Confirmation of the presence of bluetongue depends on serology, so a final result may not be available for up to four weeks after inoculation. Blood from viraemic animals from a natural transmission can be a valuable source of 'wild' virus (ie. virus that has never been passaged through cell culture, or mice or chicken embryos) for pathogenicity studies.

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Virus isolation

Virus isolation generally involves the inoculation of chicken embryos and/or cell cultures. One method uses intravenous inoculation of embryonated chicken eggs, followed by passage first in mosquito cells and then in BHK21 tissue cultures: this method has a level of sensitivity similar to that of sheep inoculation. The presence of virus in a specimen is detected by the occurrence of cytopathic effects (CPE) in the indicator (usually BHK21) cells, but needs further confirmation by antigen detection methods (including neutralisation with specific antiserum). The advantage of this method is that viable virus is available for typing to current world standards, and its virulence may also be tested by using the source material.

Methods using the direct detection of antigen in chicken embryos are of equal sensitivity and reduce the time for screening for the presence or absence of bluetongue virus. Antigen detection by enzyme linked immunosorbent assay (ELISA) has high sensitivity, but nucleic acid probes, immunostaining and 'dot blot' techniques may also be useful. Direct inoculation of specimens into cell cultures, bypassing the chicken embryo amplification step, usually has a markedly lower sensitivity.

Antigen and nucleic acid detection

Methods for the direct detection of antigen or nucleic acid in animal tissues have been developed in research projects but generally have a lower sensitivity than virus isolation, and so have not been adapted for routine diagnostic use. Antigen detection ELISA appears to show some promise in this area.

Polymerase chain reaction

The polymerase chain reaction (PCR) is the newest and most rapid method currently available to confirm early bluetongue infections by detecting viral nucleic acid. The presence of bluetongue virus in blood or tissue specimens can be proven within 36 to 48 hours. Although the technique is generally as sensitive as virus isolation, it is technically difficult, requires staff training, stringent quality control and laboratory discipline, and expensive equipment and reagents. Another disadvantage is that PCR does not distinguish between intact viable virus and RNA fragments. This is particularly important with vector studies, as the virus may be contained within the remains of a blood meal and not infective. Finding bluetongue RNA in an insect does not prove that it is a vector of that virus as the virus may not have multiplied, and residual virus may persist at low levels. The PCR technique may also be used to serotype some bluetongue viruses.

Tests for Antibodies

The requirements of satisfactory tests for antibodies are that:

- the tests should be sensitive, specific, highly reproducible, able to be standardised and evaluated internationally, and inexpensive; and
- reagents should be readily available, preferably based on non-infectious antigens, and stable after transport over long distances at variable temperatures.

Preferred tests are those that indicate the correct status of an animal soon after infection and over a long period of time. Available tests include complement fixation (CF), agar gel immunodiffusion (AGID), the competitive ELISA (cELISA) and the virus neutralisation (VN) test. The CF, AGID and cELISA tests are bluetongue group tests while the VN test is serotype-specific. The advantages and uses of these tests are as follows:

Group antibody

The *CF test* is technically complex and frequently has problems with unsuitable or anti-complementary sera. Antibodies may not be detected in this test for a relatively long time after infection (up to 45 days) and antibody is usually short lived (4–12 months). This test also has problems with a lack of specificity and many false positives are detected, especially in cattle or sheep in regions where neither bluetongue nor related viruses exist. The CF test is no longer recommended for bluetongue diagnosis, and is not routinely used in Southeast Asia, being applied only to imported animals.

The *AGID test* is cheap, simple to perform, requires minimal laboratory facilities, and can be used with poor quality sera. It detects antibodies to all viruses in the bluetongue group but, to a variable extent, also detects cross-reacting antibodies to viruses in related orbivirus groups. The reading of test results is subjective so weak positives may be missed. Antibodies develop very early and may be detected from eight days after infection, with animals almost always positive after 14 days. Antibody usually persists for at least one year in cattle after a single infection and longer in sheep. While AGID is not as sensitive as the cELISA, it is very useful for testing sentinel animal sera for the first appearance of antibodies to indicate that virus may be found in a blood sample.

The *cELISA test* uses monoclonal antibodies against a specific component of the bluetongue virion which is common to all bluetongue viruses. As these monoclonal antibodies do not react with other viruses, the cELISA test is bluetongue-specific. It is also more sensitive: antibodies may be detected in some cases from 7 or 8 days after infection and per-

sist for a very long time (perhaps many years). While cELISA is quicker than AGID, it needs specific laboratory equipment: however, as the latter is generally available in most diagnostic laboratories, it allows the test to be semi-automated and eliminates subjectivity during reading. The cELISA has been standardised and evaluated internationally and is the preferred test for bluetongue group antibodies. Kits of test reagents are available commercially.

Serotype-specific tests

Animal protection tests are the oldest serotype-specific test. They depend on the availability of susceptible sheep and the ability to reproduce disease under experimental conditions. These tests take more than one month to complete and require specific controls.

Virus neutralisation tests are based on the detection of neutralising antibodies, which are usually detected from 8 to 18 days after infection and usually persist for at least one year. Individual tests are required for each serotype: tests have been developed for all 24 serotypes of bluetongue viruses. The tests depend on cell culture, and require good laboratory facilities and good quality samples. Virus neutralisation tests generally detect antibody that is specific to a particular serotype but there can be cross-reactions between some serotypes. As there are minor variations even in viruses of the same serotype, for optimal results VN tests must be standardised for each country. However, since these tests rely on an active biological system, standardisation is more difficult than for cELISA and AGID and may become more subjective. This means that VN testing should be used for confirmatory and serotyping after the use of a group screening test such as cELISA. Nevertheless, a VN test is especially useful when applied to diagnostic sera and sera from sentinel animals where seroconversion on samples taken two to four weeks apart can identify the serotype: this kind of test is often referred to as a serum neutralisation test.

Bluetongue—Its Impact on International Trade in Meat and Livestock

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Abstract

The papers presented in these Proceedings provide strong and growing support for the concept that bluetongue disease is an endemic disease of the tropical regions when it is sustained by an active population of *Culicoides* vectors and suitable mammalian hosts. There are periodic outbreaks of bluetongue adjacent to the tropics. Countries with large populations of Merino sheep and seasonal incursions of competent *Culicoides* vectors appear to be most at risk, although other breeds can become seriously affected on occasion without any apparent reason. These Proceedings also help to clarify the disparities in the pathogenesis of the disease in different countries. These disparities may have been the result of using attenuated viruses in vaccines to control outbreaks of the disease, resulting in transmission of the attenuated vaccine viruses by the *Culicoides* vector with possible reassortment of genes between attenuated and wild viruses. It is clear that the history of viruses used in experimental disease must be well-defined as some of the international sanctions are based on information derived from laboratory altered viruses. These recent advances in the knowledge of bluetongue have provided a sound basis for a reassessment of the protocols applied by many countries against bluetongue. It now seems that the use of restrictive protocols has disadvantaged access to improved ruminant genetic material. It is anticipated that, as the result of disseminating this knowledge and its implications, less restrictive protocols will be developed among countries in the bluetongue zone, especially Australia and her trading partners, to reduce the impact of bluetongue on trade in ruminants and their products. These protocols will recognise regional and seasonal freedom of virus activity, and the limited period of viraemia when the ruminants are infectious and capable of spreading the disease. The protocols could also allow for the increased flexibility and safety that can be achieved by using core-like particles (CLPs) to protect sheep exported to countries where they may be exposed to bluetongue infection.

BLUETONGUE disease is a List A disease as classified by the Office International des Epizooties (OIE). List A diseases are defined as communicable diseases which have the potential for very serious and rapid spread, irrespective of national borders, which are of serious socioeconomic or public health consequence and which are of major importance to the international trade of livestock and livestock products. Member countries of the OIE are obliged to report these diseases, and trading partner countries are usually strict with regard to regional freedom and health certification. The 15 List A diseases include foot-and-mouth disease, rinderpest, hog cholera and Newcastle disease.

Why Is Bluetongue a List A Disease?

Bluetongue earned its notoriety based on experience with the disease in Merino sheep in South Africa and USA and, in particular, in an epidemic in Merino sheep in the Iberian Peninsula in 1956. Bluetongue's global epidemiology and effects are well covered in other papers in these Proceedings.

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Many of the present trade restrictions were formulated on the basis that some cattle remain persistently infected with bluetongue and act as symptom-free reservoirs for the disease, and that international movement of such cattle or their products, such as semen, could spread the disease. These concerns about the role of cattle as reservoir hosts and possible agents of disease spread have led virus-free countries to restrict or ban the import of ruminants from areas where bluetongue is endemic. Another factor that has fuelled this concern has been the lack of definitive information about bluetongue's epidemiology throughout the world. Some countries where certain serotypes are endemic have also been concerned to restrict imports to prevent the introduction of what they perceive as new, more virulent, serotypes.

Bluetongue—an Arbovirus Disease Endemic to the Tropics

The situation regarding the status of bluetongue disease of sheep is becoming clearer. Bluetongue can no longer be regarded as an emerging virus disease originating from Africa. As bluetongue's causal agent is an arbovirus carried by particular *Culicoides* species, it is dependent for its spread on the distribution and population fluctuations of the competent vectors among the *Culicoides* species that are present. Bluetongue now appears to be endemic in most tropical regions of the globe with periodic incursions into the subtropical and temperate regions in seasonal conditions favourable to its *Culicoides* vectors. The 24 serotypes of the virus are not uniformly distributed even in the endemic areas, and there appears to be a degree of polymorphism in genotype within these serotypes.

In China, clinical bluetongue disease has been diagnosed in six provinces since 1979 and strains of bluetongue virus (BLU) serotypes 1 and 16 have been isolated in six provinces as well as in Xinjiang and Inner Mongolia. On the basis of subsequent studies reported in these Proceedings, it is considered that bluetongue is endemic in the southeastern third of China bounded to the west by Yunnan, Sichuan, Shanxi, Henan, and Shandong Provinces with incursions as far as Inner Mongolia. There is considerable support in the Chinese Provinces for the maintenance of a series of sentinel herds to monitor the widely dispersed outbreaks of bluetongue which have occurred in the country.

In Indonesia, four sentinel herds were used to monitor bluetongue activity in West Java, Bali, Nusa Tenggara Timur and Irian Jaya. The viruses for serotypes BLU1, 3, 7, 9, 12, 16, 21 and 23 have been isolated from the blood of apparently healthy cattle and large ruminants had a higher prevalence of infection than small ruminants. Since local sheep have not shown any clinical disease, Merino sheep of a susceptible age from Australia were used to demonstrate that clinical signs were mild, indicating that isolates BLU9 and 21 were of low pathogenicity.

In India bluetongue has been recognised as endemic, after an initial outbreak in 1964. While cattle and goats have been seropositive for bluetongue, they have not shown evidence of clinical disease, although the latter has been consistently demonstrated in native and exotic sheep. Serotypes BLU1, 2, 3, 9, 16 and 23 have been identified by virus isolation while antibodies to 11 other serotypes have been recorded.

In Malaysia, bluetongue is endemic with no manifestation of clinical disease in cattle, buffalo, sheep or goats. Sentinel herds set up at four sites throughout Malaysia yielded samples from which six different serotypes were isolated; BLU1, 2, 3, 9, 16 and 23. Pathogenicity studies on Merino × Border Leicester sheep using serotypes BLU1, 2, 3, 9 and 23 demonstrated that the sheep had some degree of tolerance or resistance to the disease. It would be interesting to examine the pathogenicity of these viruses with susceptible Merino sheep.

In Thailand and Myanmar, serological testing using the agar gel immunodiffusion (AGID) test has shown that cattle, goats and sheep and group had antibodies to bluetongue. However, there has been no evidence of clinical disease in sheep, goats or cattle. There is no information on the bluetongue situation in Vietnam, Laos, Cambodia and Taiwan.

As the gaps are filled regarding bluetongue in Southeast Asia, the general situation will become clearer, but already there is strong evidence that the disease is endemic in tropical countries. This notion is supported by the knowledge that, with the exception of Indian native sheep, no local sheep display clinical signs of the disease (or, on rare occasions, display only very mild disease). Indeed, it appears that the type of sheep most susceptible to the disease are mature Merinos. Even cross-bred Merinos seem more tolerant of the disease than pure Merinos. This means that pure Merinos are likely to develop severe bluetongue signs when introduced into these endemic areas: cross-bred Merinos would also be at risk, but to a lesser degree.

There is keen interest in other Southeast Asian countries in completing the overall picture of bluetongue infection throughout the region. Vietnam, in particular, needs to establish the incidence of bluetongue in that country. Because of the major climatic and seasonal changes in Vietnam from south to north, research into the epidemiology of the virus and its vectors in that country should provide valuable additional information on bluetongue epidemiology in tropical and subtropical regions.

Insect Vectors for Bluetongue

To class an insect species as a competent vector of an arbovirus several criteria must be met:

- The insect should be shown to feed on the mammalian host.
- The virus should be shown to be ingested by the insect host, multiply in the insect and not be present in the host adventitiously.
- The insect must be shown to be able to transmit the virus biologically from one mammalian host to another.

Unfortunately, information on vector competence for bluetongue virus among *Culicoides* species is almost non-existent in the Asian-Pacific region, with the exception of Australia and some data from Indonesia and Malaysia.

Using light traps, Indonesian workers established that the vectors of bluetongue known on the basis of Australian experiments (*C. brevitarsis, C. actoni, C. wadai* and *C. fulvus*) were present in significant numbers at several of their trapping sites. Subsequently BLU21 was isolated from *C. peregrinus* from a mixed sample of *C. fulvus* and *C. orientalis*, and from a sample of *Anopheles* species while BLU1 was isolated from *C. fulvus*.

In Malaysia, on the basis of observed abundance, distribution and host preference, *C. peregrinus, C. orientalis* and *C. shortii* may be considered possible vectors for BLU viruses. However, in Australia these three criteria alone have not been found sufficient for defining a competent vector. The main criterion that must be met is the insect's capacity to transmit virus from one mammalian host to another. Further studies need to be carried out in Malaysia to establish the competent vectors there.

More detailed information is needed on the distribution and competence of *Culicoides* as vectors of bluetongue in the Asian-Pacific region and their seasonal fluctuations. Until this is done, there will be only an incomplete understanding of the epidemiology of the disease in the region on which to base risk assessment.

Pathogenesis of Bluetongue and Its Variability

As explained earlier, the classical pathogenesis of bluetongue has been based on experience with the disease in South Africa and USA and the 1956 outbreak in the Iberian Peninsula.

Papers in these Proceedings challenge these views. Australian research, using field or 'wild' viruses that had not been processed or adapted in the laboratory, showed that serotypes BLU3, 15, 16 and 23 were highly virulent to older Merino sheep, producing clinical signs equal to those seen in the most severe bluetongue infections and mortality rates from 8%–32%. While infections with these bluetongue serotypes were severe, the disease was short-lived, the virus did not cross the placenta to cause foetal deaths or abnormalities, and virus was not found in semen (except occasionally during viraemia of relatively short duration). While cattle showed no clinical disease symptoms, they did become viraemic, and occasionally bulls excreted virus in semen during viraemia.

In contrast to 'wild' viruses, Australian serotypes attenuated for vaccine experiments have been shown to be teratogenic, in a similar fashion to attenuated South African and North American serotypes, if administered to ewes during the first half of pregnancy. Attenuated virus strains have been transmitted by *Culicoides* vectors. Since BLU viruses readily recombine with each other, it is reasonable to assume that attenuated viruses could recombine with wild type virus to create a new virus with characteristics of both.

US research presented in these Proceedings compared the BLU10 field isolates from 1980–81 and 1990 with prototype BLU2, 10, 11, 13 and 17 and vaccine virus by sequence analysis. This suggests field isolate BLU13 may have been derived in part from a reassortment virus which has a gene segment from the BLU10 vaccine virus. This evidence illustrates the genetic polymorphism of the BLU viruses, since their replication cycle involving insect vectors and mammalian hosts offers opportunities for genetic reassortment and the development of new viruses. It also highlights the risks associated with the use of an attenuated bluetongue vaccine during an active outbreak of bluetongue whereby wild viruses may acquire the undesirable teratogenic characteristics of the attenuated vaccine virus.

Studies in the USA on the virus amplification that occurs before transmission of BLU to *Culicoides* vectors showed that the virus becomes associated with blood cells, resulting in a prolonged viraemia of up to 50 days approximating the infectivity period. Viral nucleic acid can be detected for up to 150 days in cattle and for slightly less time in sheep, a period that approximates the life span of the red blood cell.

Bluetongue as a Barrier to Trade in Livestock

As described earlier, various concerns about the role of cattle as reservoir hosts for bluetongue and the lack of definitive information about bluetongue epidemiology worldwide have led to trade restrictions among countries.

The accumulated research data indicates that bluetongue virus causes an infection that is variable in severity but discrete in duration: after this time the ruminant is no longer infected although still retains antibodies to bluetongue. The use of attenuated vaccines in some countries has complicated the situation in those countries in terms of the nature of the disease. However, ruminants that show antibody to bluetongue as the result of natural infection or vaccination and are no longer viraemic should not pose a risk of causing an outbreak of the disease when introduced into other areas. Nevertheless, these animals may become infected by the local serotypes of bluetongue. When this does occur, the available evidence suggests that the severity of the subsequent infection is reduced and mortalities prevented.

There is unlikely to be any threat of disease outbreak posed by the international movement of semen and embryos from cattle, sheep or other ruminants which are seropositive to bluetongue but were not viraemic at the time of collection of semen or embryo.

Vaccination as a Means of Facilitating International Movement

Attenuated live virus

Attenuated vaccines have been used extensively in South Africa and the USA with considerable success in controlling clinical outbreaks of the disease. These vaccines are the cheapest to produce and provide a high level of protection although, as discussed earlier, they do have some associated problems. There is no doubt that South African and North American vaccine strains are teratogenic. While the relative level of teratogenicity of attenuated virus from South African and Australian serotypes is the subject of contention, the risk involved in the use of attenuated Australian serotypes has been clearly established in one Australian laboratory. The other factors of genetic recombination and transmission by *Culicoides* species provide further constraints against using attenuated vaccines to control outbreaks.

Nevertheless, such vaccines may be of value in protective vaccination of ruminants for export to countries where bluetongue may be endemic, provided that the vaccination is carried out in the absence of vectors and 150 days before export. Even then, there would always be a risk that the vaccine could be used more widely without precautions taken to prevent infecting vectors. The resultant recombinants with wild strains could then infect *Culicoides* vectors. Overall, therefore, it would wisest not to use attenuated vaccines at all.

Inactivated whole virus vaccine

An inactivated whole virus vaccine is more costly to produce than an attenuated live virus vaccine but has been demonstrated to provide protection against experimental infections with virulent virus. While there may also be some level of protection against challenge with some heterologous serotypes, this has not yet been well documented. The success of inactivated vaccines in China has been described.

Virus-like particles (VLPs) vaccines

VLPs contain the core proteins as well as the outer capsid proteins. As VLPs carry no viral genetic material, they possess the advantage of being non-infectious. VLPs have been shown to immunise sheep: the immunity is generally serotype-specific and confers some heterologous protection.

Core-like particles (CLPs)

CLPs comprise the core proteins of bluetongue virus and are easier to produce than VLPs. In separate South African and Australian experiments, CLPs produced partial immunity in sheep challenge with wild viruses. This partial immunity was not serotype-specific and may be largely cell-mediated. Although further work is necessary to determine the value of CLPs as a vaccine, they may have promise in conferring short-term immunity on animals that are to be imported into countries where bluetongue is endemic. Certainly CLPs could be examined as a means of protecting sheep imported into the southeastern sector of China, which appears to be an endemic zone.

Commercial Production of a Vaccine

A major factor in the production of vaccines against bluetongue has been the commercial aspects of vaccine manufacture. Most manufacturers need a demonstrated market before they are prepared to invest capital and other resources into vaccine production. The situation in South Africa has produced such a market for their cheap attenuated bluetongue vaccines because of the continual threat to their sheep population of the multiple serotypes of bluetongue. However, in many other countries, such as Australia, bluetongue vaccines are seen as a means of containing an outbreak of the disease which may never occur. In such circumstances it is not economic for a manufacturer to develop or produce vaccines. As all vaccines other than the CLPs are serotype-specific, it has been accepted that vaccines would be needed against each serotype. CLPs present an opportunity to produce a vaccine which could have a use in international trade as well as providing a level of protection in the event of an outbreak. CLPs can be used at any time, and can be modified so that vaccinated and

bluetongue-infected sheep can be differentiated by the use of an appropriate ELISA test.

Conclusion

In the Asian-Pacific region almost all countries have endemic bluetongue with multiple serotypes. The probability is that more serotypes will be found although their individual distributions are presently poorly understood. The current trade sanctions are based on experimental data that is now known to have been inaccurate. The information presented in these Proceedings is a useful basis on which to reassess the rules relating to bluetongue in this region, for mutual benefit, without increasing risk.

Future Directions in Bluetongue Research

P.D. Kirkland*

UNTIL very recently, we had very little information on bluetongue viruses in China, but these Proceedings show how the situation has changed. We have had many interesting papers, ranging from studies of disease outbreaks in China and other countries to research involving advanced molecular biology techniques. At the Symposium it was valuable to have discussions involving scientists from so many countries in this Asian-Pacific region. There are many young scientists who now have training in a wide range of bluetongue research methods, so consequently there are many opportunities to make rapid progress in bluetongue research in this region.

However, the starting materials for our projects usually comprise blood and tissue samples from animals, and subsequently the viruses amplified in the laboratory. In some Asian countries at present, it is difficult to collect a large number of samples from animals. Close cooperation is needed among field veterinarians, farmers, laboratory researchers and government officials to ensure that specimens are readily available in the future. When this happens we are sure to see even more rapid progress in bluetongue research in the Asian-Pacific region. With rapid transport available, collaboration between countries is easier. We have the possibilities of exchanging information and sharing valuable laboratory resources through close collaboration. I look forward to hearing the results of successful studies in the future.

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The First Southeast Asia and Pacific Regional Bluetongue Symposium

Closing Address

Zhao Shi Kun*

LADIES and gentlemen—the First Southeast Asia and Pacific Regional Bluetongue Symposium is about to be closed. I am summarising this Symposium on behalf of Mr Lin Wenlan, the Director of Yunnan Provincial Commission on Science and Technology and the Chairman of the Management Committee of the Yunnan Tropical and Subtropical Animal Virus Disease Laboratory. The 105 delegates at the Symposium have come from Australia, the United States of America, United Kingdom, Vietnam, India, Indonesia, Malaysia, Philippines, Thailand, Papua New Guinea and Japan (11 countries in all) and also from the Department of Animal Industry and Veterinary Medicine, the Department of International Cooperation and Animal Quarantine Institute of Ministry of Agriculture, China, and from 10 provinces (Yunnan, Hubei, Anhui, Inner Mongolia, Xinjiang, Guangxi, Jiangsu, Shanxi and Gansu).

The participants exchanged their experience in the aetiology, epidemiology, diagnostic methods, immunology, control and entomology of bluetongue, and the desired results have been achieved.

- We have a clear understanding of the epidemiology and the distribution of bluetongue in the Asia and Pacific Region.
- There is some progress in the aetiology.
- We note that some gratifying achievements have been made in the area of vaccines and control, especially attenuated vaccines, inactivated vaccines and genetically-engineered vaccines. Of these, some vaccines have been used in the animal industry.

Throughout the Symposium, we have noted great successes in research on bluetongue in Asia and the Pacific region. The success displayed will further promote research and control of bluetongue and strengthen the connection and academic exchanges among the scientists in this region, so this is a successful Symposium.

This success would not have been possible without the attention and support of Agriculture Ministry, People's Republic of China, the Yunnan Provincial Government, the Australian Centre for International Agricultural Research and the Yunnan Tropical and Subtropical Animal Virus Disease Laboratory working together. The Symposium Committee and the scientific staff of Yunnan Tropical and Subtropical Animal Virus Disease Laboratory have also devoted months to the complex preparation necessary for the success of this Symposium. All the participants here have also taken full advantage of the opportunity for discussions.

The Symposium was held here in the Greenlake Hotel which provided such good services and facilities. This meant that the delegates had more time to exchange experience and to establish friendship with each other outside the formal sessions. This had the two-fold effect of promoting information exchange and friendship.

Science and technology is the wealth shared by human beings. The achievements will be used to serve the people in all the world. I hope the achievements in this Symposium, the knowledge, wiil also become the wealth shared by us all. The Symposium is closing, but it will be taken as a point to accelerate research of bluetongue in the world.

Now I declare the First Southeast Asia and Pacific Regional Bluetongue Symposium closed! I wish everybody good health and a safe trip home. Thank you.

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