final report

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Prepared by: Dr Lorna Melville & Dr Peter Kirkland
Northern Territory Department of Primary Industries & Fisheries

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Evaluation of Bluetongue Virus Excretion in the Germplasm of Cattle

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FINAL REPORT FOR NTA 018 - PART ONE

ABSTRACT

The aims of this project were to determine the factors involved in the contamination of semen with bluetongue viruses and to develop a safe protocol for the export of semen from previously infected animals.

Experiments were conducted to monitor the duration of viraemia and excretion of virus in semen in both naturally and experimentally infected bulls.

In the Northern Territory, bulls were exposed to natural infection with bluetongue viruses over three wet seasons, 1991-1993. During these years bulls were infected with BLU 1, BLU 3, BLU 16 and BLU 20.

Serological monitoring of sheep inoculated with semen samples collected during the observation period failed to indicate any contamination of semen with these viruses.

In NSW, frozen semen samples were examined from a group of mixed age bulls which were naturally infected with BLU 1. There was no evidence of bluetongue virus in any of the semen samples collected around the period of viraemia.

Experiments were conducted to examine the effect of age of bulls, laboratory adaptation of virus and bluetongue virus serotype on the excretion of virus in semen. Groups of 5-8 young bulls (2-4 years) and mature bulls (5-15 years) were inoculated with either "wild" virus or laboratory adapted virus. Two serotypes were used: BLU 1 in NSW and BLU 23 in the Northern Territory.

When mature bulls were inoculated with laboratory adapted virus all bulls became viraemic and virus was detected in the semen of several bulls with both serotypes 1 and 23. When mature bulls were inoculated with "wild" virus, only serotype 23 infected bulls excreted virus with semen. This may have been the result of blood contamination of semen in the aged bulls used in this experiment.

When young bulls were inoculated with "wild" virus or laboratory adapted virus, no evidence of virus contamination of semen was found with either serotype 1 or 23.

To examine bulls for latent infection with bluetongue viruses, seropositive bulls were immunosuppressed, then blood and semen samples were checked for bluetongue viruses by sheep inoculation. Seropositive bulls were also slaughtered and tissues collected from the spleen and reproductive tract. These were then checked for the presence of virus. No evidence of latent infection was found.

From these findings a practical protocol was developed to allow the collection of semen from bluetongue seropositive bulls. If a bull is held in a vector free environment for a sufficient length of time to extend beyond any likely period of viraemia, there should be no risk of the semen containing bluetongue virus.

FINAL REPORT FOR NTA- 018 PART TWO

1.BACKGROUND AND INDUSTRY CONTEXT

With the refinement of artificial breeding techniques for cattle, there is now greater emphasis on the international movement of frozen semen, ova and embryos as a means of introducing new genes to the cattle population. These artificial breeding techniques are also generally seen as a method of providing disease-free germplasm from animals which have been or are actively infected with exotic micro-organisms. In Australia, substantial trade has developed from the export of semen and embryos from genetically superior cattle. In addition to the income generated from these export markets, there has also been significant gain to some producers following the introduction of new breeds or blood lines to Australia and, often, their subsequent export to other trading partners.

The greatest impediment to this valuable export market has been the presence in Australia of several pathogenic arboviruses, especially bluetongue. In Australia each year, loss of export income amounting to many millions of dollars occurs when animals are found to be serologically positive to bluetongue. It is known that bluetongue virus can be excreted in the semen of experimentally infected bulls in the immediate post-infection period, especially while the bull remains viraemic, even in the presence of circulating antibody. The duration of this virus excretion is not known but can be at least 30 days after the onset of viraemia. The report of a single persistently viraemic bull in the USA has to date prevented the acceptance of an upper limit for the duration of virus excretion in semen, so much so that semen exports from bluetongue seropositive bulls are generally prohibited. Overseas it has been reported that foetuses may be infected in utero, with some infections resulting in a persistently infected, immuno-incompetent calf.

The purpose of this project was to investigate the likelihood of bluetongue virus contaminating bull semen and to develop a safe protocol for the export of semen from previously infected animals.

2.PROJECT OBJECTIVES

(i)To establish, in bulls, the relationship of viraemia to the shedding of virus in the semen.

(ii)To determine whether, after cessation of viraemia, semen samples from serologically positive bulls contain bluetongue virus and whether reliable procedures are available to certify semen as free of bluetongue viruses.

(iii)To determine whether bluetongue seropositive bulls are latent carriers of the virus either in the reproductive tract or systemically.

3.METHODOLOGY

Experiments were conducted to monitor the duration of viraemia and excretion of virus in semen in both naturally and experimentally infected bulls.

(i) **Natural infection**. Each year in January from 1991-1993, a group of 10 seronegative young bulls was introduced to Coastal Plains Research Station (CPRS) near Darwin. Bulls were bled and ejaculated twice weekly from January - June. Whole blood and semen samples were collected and examined for virus contamination by virus isolation and sheep inoculation. Sera from the bulls and the inoculated sheep were checked monthly for seroconversion to bluetongue.

In NSW, frozen semen samples were examined from a group of mixed age bulls which were naturally infected with BLU 1. A total of 130 batches of semen were collected from 19 bulls during the period of viraemia and up to 3 months later. All semen samples were examined by sheep inoculation and monitoring antibody responses of those sheep.

(ii) **Experimental infection**. A trial was conducted to examine the effect of laboratory passage of virus, age of bulls and bluetongue virus serotype on the excretion of virus in semen.

Groups of 5-8 young bulls (2-4 years) and mature bulls (5-15 years) were selected from bluetongue free areas and inoculated with either "wild" virus or laboratory adapted virus. Two serotypes were used: BLU 1 in NSW and BLU 23 in the NT. These serotypes were selected for study because BLU 1 is the Australian serotype most likely to infect bulls eligible for entry to AI centres, and BLU 23, currently restricted to the NT, is the most pathogenic Australian serotype.

(iii) **Examination of bulls for latent infection**. Seropositive bulls were immunosuppressed using corticosteroids, then blood and semen samples were checked for bluetongue viruses by sheep inoculation. Seropositive bulls were also slaughtered and tissues collected from the spleen and reproductive tract. These were checked for the presence of virus by sheep inoculation.

4.RESULTS AND CONCLUSIONS

(i) **Natural Infection**. During the three years 1991-1993, bulls at CPRS were naturally infected with a number of bluetongue serotypes.

In 1991, one bull was infected with BLU 3, in 1992 ten bulls were infected with BLU 16 and in 1993 ten bulls were infected with BLU 1 and one with BLU 20. All bulls were shown to be viraemic but virus was not detected in any of the semen samples by sheep inoculation.

In NSW, there was no evidence of bluetongue virus in any of the 130 batches of semen collected from 19 bulls during the period of viraemia and subsequently.

(ii) **Experimental Infection**. When young bulls were inoculated with "wild" virus or laboratory adapted virus all bulls became viraemic, but no evidence of virus contamination of semen was found with either serotype 1 or 23.

When mature bulls were inoculated with laboratory adapted serotype 23 all bulls became viraemic and virus was detected in the semen of 3/5 bulls during the period of viraemia but never after the cessation of viraemia. When mature bulls were inoculated with "wild" serotype 23 all bulls became viraemic and virus was detected in the semen of 3/5 bulls during the period of viraemia. Four ejaculates at the peak of viraemia showed visible contamination with blood.

When mature bulls were inoculated with laboratory adapted serotype 1 all bulls became viraemic and virus was detected in the semen of 5/8 bulls. In 2 of these 5 bulls virus was detected in the semen only during the period of viraemia. Virus was detected in the semen of the other 3 bulls for up to 10 days following the period of detectable viraemia. Although semen collections from the 5 bulls continued for a further 12 weeks, virus was not again detected. When mature bulls were inoculated with "wild" serotype 1 all bulls became viraemic but no virus was detected in semen.

(iii) **Latent Infection**. There was no evidence of bluetongue in blood or semen of immunosuppressed bluetongue seropositive bulls. Similarly, tissues from the reproductive tract and spleen failed to show any evidence of bluetongue viruses when inoculated into sheep.

These results suggest that the use of bluetongue virus which has been adapted to grow in cell culture may be a major factor contributing to the contamination of semen in experimental infection. The presence of virus in the semen of old bulls inoculated with "wild" virus appeared to be associated with the contamination of semen by blood.

In contrast to the results with experimentally infected bulls, there was no evidence of bluetongue virus in the semen of any of the 41 bulls which had been naturally infected. The younger age of these bulls may also have been a significant factor contributing to the lack of contamination of semen with virus.

It would appear that contamination of bovine semen with bluetongue virus following natural infection is relatively uncommon and could be related to contamination of the

semen of older bulls with blood or leucocytes. There is no evidence to suggest that semen is contaminated with bluetongue other than immediately around the period of viraemia, or that a latent infection state exists in seropositive bulls. These findings suggest that a practical protocol can be developed for the collection of semen from seropositive bulls.

5. RECOMMENDATIONS

The results of these studies show that when bulls have been infected with "wild" virus, there is little risk of virus being found in semen at any time.

When live virus vaccines are used for bluetongue, there is a risk of insect transmission of vaccine virus and there may be a short time during, or soon after, the period of viraemia when virus may be found in semen. In Australia, however, in the absence of vaccines for bluetongue, there appears to be minimal risk of the excretion of virus in bull semen. At worst, virus is only ever likely to be found in semen during or soon after the period of viraemia, for a maximum of about 40 days after infection. As it is difficult to define the precise time of infection of bulls in the bluetongue endemic areas, especially if animals have already been infected with one serotype and are seropositive, it may be necessary to define a longer qualifying period before bulls are eligible to provide semen for export. In practical terms it will be necessary to move bulls to a vector free region, hold them for a minimum of 3 months and then it will be absolutely safe to collect semen for export to bluetongue-free countries. Although most countries will accept semen from bulls which are seronegative, sometimes there are stringent additional requirements for confirmatory virus isolation on semen if the bull originates from a vector region. The previously described quarantine conditions would be applicable to bulls regardless of their serological status for bluetongue and will allow unrestricted export of bovine semen without the need for expensive, testing of both blood and semen.

The proposed quarantine conditions have been discussed on several occasions with AQIS personnel. It is encouraging to report that new protocols are currently being drafted for the importation of bovine semen from the USA, initially from seronegative bulls at any time of the year and eventually also from seropositive bulls. With a significant relaxation of conditions for importation into Australia, more rational conditions for the export of semen from Australia can be expected. Nevertheless every opportunity should be taken to promote the significant outcome of this project to ensure maximum benefit for industry. It is particularly important that the conservative EEC countries become aware of and accept these results and the logical export certification requirements that would follow. Fortunately, there have been recent opportunities in this direction. Representatives of the Veterinary Commission of the EEC visited Australia during 1994 to discuss progress with disease monitoring programs, recent research results and to inspect laboratory facilities. During this visit Dr Kirkland was able to present at some length the results of this project to the Commissioners who responded very positively.

FINAL REPORT FOR NTA- 018 PART 3

1.BACKGROUND AND INDUSTRY CONTENT

With the refinement of artificial breeding techniques for cattle, there is now a growing emphasis on the international movement of frozen semen, ova and embryos as a means of achieving best use of superior genetic strains for livestock improvement programs.

Australia is at the forefront in the use of artificial breeding and quarantine procedures to introduce, sample and utilise superior livestock strains from overseas countries. A recent example is the introduction of the Boran and Tuli bloodlines from East Africa to Australia.

Cattlemen in Australia have also earned a world reputation for practical blending of different cattle characteristics to produce animals which will survive and produce under a wide range of conditions. New breeds such as the Droughtmaster, Belmont Red, Braford, Brangus and Murray Grey provide commercial cattlemen with a range of genetic options for their environment and beef market. Australia has successfully produced two new tropical dairy breeds which are attracting increasing demand for livestock development programs in countries where temperate dairy breeds perform poorly.

The development and adoption of new selection technology such as "Breedplan" and the Australian Dairy Herd Improvement Scheme has ensured that the Australian cattle industry is in a position to support its export industry with objective and comparative production data.

World genetic linkages of cattle populations, demonstrated in both the dairy and beef cattle industries, have allowed the identification of "super sires" which are in demand internationally and can earn in excess of 5 million dollars over one or two breeding seasons. Individual Australian sires have earned between 1-2 million dollars in the domestic market.

In the process of developing its cattle industry, Australia has been a gross importer of genetic material through artificial insemination and live cattle imports. During the 1980's, Australia has imported over 3 million doses of semen, mainly from Canada, United States, United Kingdom and New Zealand. Exports during that period have been less than 500,000 doses, with the majority to New Zealand and South East Asia.

The greatest impediment to the development of a substantial export market of Australian cattle genes is the presence in Australia of several pathogenic arboviruses, particularly bluetongue. Much of Australia's cattle industry operates within the reach of the vectors which can transmit arboviruses and considerable national effort has been made to study the variety and range of viruses and vectors.

An increasing number of Australia's customers and potential customers are becoming

concerned about introduction of new viruses or virus strains to their countries and are adjusting their import protocols accordingly. Countries which require bluetongue test freedom of donor sires include United States, Canada, most EEC countries, Argentina, Brazil, Uruguay, Mexico, New Zealand, Taiwan, China, Burma, Thailand and Korea.

Included are a number of our near Asian and Pacific neighbours who are high priority trading partners for Australia's livestock industry. In these countries, semen and embryos often allow a sampling of Australian genetic material prior to the development of more substantial livestock breeding programs, and in many cases, the concurrent introduction of processed livestock products.

The current loss to Australia of potential artificial breeding trade exceeds 10 million dollars per year. The loss to the industry of opportunities to develop trade of breeding and commercial stock as well as associated processed products is much more substantial.

Restrictions on the use and export of semen from bulls which have been infected with bluetongue viruses have largely come about because of the findings of Luedke and associates in the USA. They reported that a bull was intermittently viraemic for many years. Combined with the knowledge that viraemic bulls may excrete virus in their semen, most countries prohibit the importation of semen from bulls which may have been infected with bluetongue viruses. Since bluetongue virus was recognised in Australia in 1977, there has been a significant loss of export income for the cattle industries because many valuable bulls have been infected with bluetongue viruses. Previous research has shown that bluetongue viruses may be found sporadically in the semen of infected bulls and only during the period of viraemia for up to 28 days. However, these studies have only used bulls experimentally infected with laboratory adapted virus and have mostly used US serotypes. Results have also suggested that only aged bulls shed bluetongue viruses in their semen. Furthermore, extensive research has provided little evidence of other cases of immunotolerance or persistence of bluetongue viruses in cattle.

In view of the results of previous experiments on the excretion of bluetongue viruses in bovine semen, the history of the strain or type of virus responsible for the infection, age of bulls, ejaculation technique and frequency of collection are all possible factors which may affect the shedding of bluetongue in semen. Sensitivity of isolation procedures may also contribute to the detection of bluetongue in bovine semen. Attempts were made to obtain data on these factors using 2 serotypes of bluetongue present in Australia. With an improved understanding of the factors involved in the excretion of bluetongue in semen, it should be possible to develop less restrictive export protocols and facilitate the export of semen from Australian bulls.

2.PROJECT OBJECTIVES

The objectives of these studies were to:

(i)Establish the relation between the occurrence of viraemia and the excretion of virus in semen;

(ii)Determine whether there is a state of latent infection with bluetongue virus and if there can be a resurgence of viraemia.

3.METHODOLOGY

Preliminary studies at Darwin showed that young naturally infected bulls did not excrete virus in their semen even during the viraemic stage. This conflicted with the literature in which it was reported that experimentally infected bulls excreted virus in their semen when viraemic.

Experiments were conducted to monitor the duration of viraemia and excretion of virus in semen in naturally and experimentally infected bulls.

(i) **Natural infection**. Each year in January from 1991-1993, a group of 10 seronegative young bulls was introduced to Coastal Plains Research Station (CPRS) near Darwin. Bulls were bled and ejaculated twice weekly from January - June. Whole blood and semen samples were collected to detect virus by virus isolation and sheep inoculation. Semen samples were diluted in an equal volume of heart brain broth with antibiotics (1500 units of penicillin and 5 mg of streptomycin per ml). Samples were split and inoculated to two sheep. A third sheep was inoculated intravenously with 5 ml of whole blood from the same bull. Blood samples were also processed for virus isolation in the laboratory. Sera from these bulls and sheep were checked monthly for seroconversion to bluetongue.

During the course of annual certification of bulls located at a commercial A.I. centre in NSW, it was found that a number of bulls had become infected with bluetongue due to an unusual extension of virus transmission south of the virus endemic area. The precise time of bluetongue infection for individual bulls was unknown, however monitoring of nearby sentinel cattle indicated that natural infection with BLU 1 occurred during April 1989. Regular semen collections had been made during the period of likely virus transmission. Samples of semen from 19 infected bulls were obtained from collections made during April 1989 and the subsequent 3 months. These collections were chosen to obtain semen collected during the period of likely viraemia and the subsequent 2 to 3 months.

Semen was available from 19 mature bulls, which had been infected, as determined by a positive result in the AGID test for bluetongue. The semen had been collected commercially from these bulls by ejaculation into an artificial vagina and graded according to sperm concentration and motility. The semen was then appropriately diluted in cryopreservatives and stored in liquid nitrogen. Ten straws (0.2 ml each) of semen were screened from each batch. A total of 130 batches of semen (1300 straws) were tested for the presence of virus. This represented varying numbers of collections

from the bulls.

Healthy sheep (260) of mixed age, sex and breed, free of bluetongue antibody at the time of inoculation were used. Five straws of semen from each batch were initially thawed at 37^{o}C (yielding 0.7 - 1 ml) for inoculation of the sheep. Two separate batches of semen from the same bull were inoculated subcutaneously concurrently into a sheep. Clotted blood was collected from the sheep prior to inoculation and then weekly for 5 weeks. This serum was then tested for the presence of antibodies to bluetongue by the AGID test and ELISA. This procedure was then replicated with the remaining 5 straws of semen per batch to confirm any initial findings. In total, approximately 1.5 to 2 ml of semen was examined from each batch. This was a similar volume of semen to that examined in the experimentally infected bulls.

(ii) **Experimental infection**. A trial was conducted to examine the effect of laboratory passage of virus, age of bulls and bluetongue virus serotype on the excretion of virus in semen.

Bull inoculation

Groups of 5-8 young bulls (2-4 years) and mature bulls, aged 5-15 years, were selected from bluetongue free areas and inoculated with either "wild" virus or laboratory adapted virus. Two serotypes were used: BLU 1 in NSW and BLU 23 in the NT. These serotypes were selected for study because BLU 1 is Australian serotype which is the most likely to infect bulls eligible for entry to AI centres and BLU 23, currently restricted to the NT, is the most pathogenic Australian serotype.

Bowen in the USA was able to isolate bluetongue virus from the semen of bulls experimentally infected with US serotypes of bluetongue virus, so efforts were directed towards reproducing similar conditions in these studies. In particular, bulls were inoculated with high titres of virus (6.5 $\log_{10}$/bull for serotype 1 and 5.1 $\log_{10}$/bull for serotype 23.

Laboratory-adapted Virus

The laboratory-adapted strains of BLU 1 and 23 used in this experiment were the master seed viruses for the Australian attenuated BLU 1 and 23 vaccines which had undergone 20 passages in BHK_{21} cells at AAHL. The inoculum for the bulls was generated by the amplification of the attenuated vaccines by a further 2 passages in BHK_{21} cells. This virus stock was stored at 4^{o}C. The bulls were inoculated subcutaneously with 10 mls of BLU 1 culture medium (a total of 6.57 $\log_{10}TCID_{50}$) or intravenously with 10 mls of BLU 23 (a total of 5.1 $\log_{10}TCID_{50}$).

"Wild" Virus

The inoculum for the "wild" virus infections was passaged so that a titre of virus similar to that used for the laboratory adapted virus was obtained. This allowed for a confident comparison between the inoculation of laboratory-adapted or "wild" virus of bulls and the effect of attenuated virus on seminal shedding. The titre of the original "wild" virus

stock was increased by passaging 1-2 times in a calf and 1-2 times in a sheep.

Each bull was inoculated subcutaneously with 100 ml of sheep blood ("wild" BLU1, 6.29 $log_{10}CEID_{50}$) or intravenously with 100 ml of sheep blood ("wild" BLU 23, 5.4 $log_{10}TCID_{50}$).

Collection of Samples

In order to monitor the presence of virus in the blood (viraemia) and the development of an antibody response, heparinised and clotted blood samples were collected each time semen was collected. Collection of heparinised and clotted blood and semen began on day 7 post inoculation, continued twice a week for 4 weeks and then once a week for 4 weeks.

Semen - Semen was collected by standard electroejaculation techniques by an experienced operator. Once the semen was collected, it was dealt with in 3 ways:

(i) 2mls of unprocessed (raw) semen was held for sheep inoculation.
(ii) 5mls of raw semen was diluted in an equal volume of foetal calf serum, aliquotted into 1ml lots, snap frozen and then stored at -80^{o}C for virus titrations and further virus isolations.
(iii) The remaining raw semen was scored for sperm concentration and motility then diluted accordingly in a cryopreservative and aliquotted into straws for long term storage in liquid nitrogen.

Blood - Blood samples for serology were collected in plain evacuated rubber stoppered glass tubes (10ml). Serum was removed from the blood clot and stored at -20^{o}C. Serum samples were tested by AGID and ELISA to detect seroconversion to bluetongue group viruses.

Blood samples for virus isolation were collected in evacuated glass tubes (10mls) with heparin added to prevent clotting. This blood was stored at 4^{o}C.

(iii)**Examination of bulls for latent infection**. Seropositive bulls were immunosuppressed using corticosteroids (dexamethasone 5mg/ml) at 25 mg daily for 5 days. Blood and semen samples were collected once per week for 4 weeks and checked for bluetongue viruses by sheep inoculation. Seropositive bulls were also slaughtered and tissues collected for virus isolation and histological examination.

Tissues included:

1.right testicle (upper and lower portion)
2.left testicle (upper and lower portion)
3.tail of right epididymis
4.tail of left epididymis
5.head of right epididymis
6.head of left epididymis
7.right seminal vesicle

8.left seminal vesicle
9.right ampulla
10.left ampulla
11.prostate
12.spleen

After collection the tissues were stored at -80^{o}C until the time of processing. Processing of the tissues for bluetongue isolation involved the preparation of a 20% homogenate (w/v) of the thawed tissue in PBS with gelatine (PBGS) and antibiotics (1000 units of penicillin, 1000 units of streptomycin and 5ug of fungizone were added per ml). This was then centrifuged at 1000 x g for 10 mins at 4^{o}C and the supernatant removed. This supernatant (2mls) was used as the inoculum for sheep.

(iv)**Virus Isolation**. Whole blood (2-5 ml), semen samples (2-2.5 ml) and homogenised tissue samples were inoculated into separate sheep. The sheep were bled at the time of inoculation and 21,28 and 35 days post inoculation for serology. Sera were tested by the AGID and cELISA to detect antibodies to bluetongue. The presence of virus in a sample was indicated by seroconversion of the recipient sheep. Blood and semen samples were also inoculated intravenously (I/V) into 11 day old embryonated chicken eggs. The embryos were homogenised and the clarified supernatant inoculated onto *Aedes albopictus* cell cultures with up to 3 further passages on BHK_{21} monolayers to detect virus.

(v)**Serology**. Serum samples from all bulls (naturally and experimentally infected) and recipient sheep were tested for antibodies to bluetongue group antigens by agar gel immunodiffusion (AGID) and competition ELISA (cELISA) and for type specific antibody by virus neutralisation (VN) test. The AGID, cELISA and VN tests were performed by standard methods as described in the Australian Standard Diagnostic Techniques.

4.FINDINGS AND CONCLUSIONS

(i)**Natural Infection**. During the three years 1991-1993, bulls at CPRS were naturally infected with a number of bluetongue serotypes.

In 1991, one bull was infected with BLU 3, in 1992 ten bulls were infected with BLU 16 and in 1993 ten bulls were infected with BLU 1 and one with BLU 20 as shown in table 1. All sheep receiving blood from infected bulls seroconverted to the corresponding virus, but no sheep receiving semen seroconverted as shown in table 2.

In NSW, there was no evidence of bluetongue virus in any of the 130 batches of semen collected from 19 bulls around the period of viraemia and stored frozen in liquid nitrogen.

Table 1.Natural Infection of Bulls

Year	Virus	No. of bulls infected By virus isolation	No. of bulls infected By seroconversion
1991	BLU 3	1	1
1992	BLU 16	10	10
1993	BLU 1	10	10
	BLU 20	1	1

Table 2.Seroconversion of sheep receiving blood or semen of naturally infected bulls

Virus	Sheep inoculated with blood		Sheep inoculated with semen	
	No. inoculated	No. infected	No. inoculated	No. infected
BLU 3	1	1	2	0
BLU 16	10	10	20	0
BLU 1	10	10	20	0
BLU 20	1	1	2	0

(ii)**Experimental Infection**

Serotype 23 in mature bulls (6 - 15 years).

Laboratory Adapted Virus: All 5 bulls became viraemic with the maximum period of viraemia being 27 days (Table 3). Virus was detected in the semen of 3 bulls during the period of viraemia (1-3 ejaculates) but never after the cessation of viraemia. Three bulls died during the observation period from stress and possible bovine ephemeral fever.

An unexpected finding was severe clinical bluetongue disease in a number of inoculated sheep. Two blood recipient sheep died with clinical and necropsy findings consistent with bluetongue disease and BLU 23 was isolated from both animals.

"Wild" Virus: All 5 bulls became viraemic with the longest duration of viraemia being 27 days. Virus was detected in the semen of 3 bulls during the period of viraemia (1-2 ejaculates). Four ejaculates at the peak of viraemia showed visible contamination with blood. Three of these produced bluetongue antibodies in inoculated sheep. Inoculation of the fourth blood contaminated sample did not cause seroconversion.

A number of inoculated sheep showed clinical bluetongue disease, but all recovered.

Serotype 23 in young bulls (2-3 years).

Laboratory Adapted Virus: All 5 bulls became viraemic with the maximum period of viraemia being 38 days. All semen samples were negative for virus when inoculated into sheep.

"Wild" virus: All 5 bulls became viraemic with the maximum period of viraemia being 31 days. All semen samples were negative for virus when inoculated into sheep.

Table 3.Detection of BLU 23 in the semen of experimentally infected bulls.

Bull	Age (years)	Duration of viraemia (days)	Virus	Ejaculates with BLU 23/ ejaculates tested
1	14	16	Lab.[1] BLU 23	1/10
2	14	16*	"	0/5
3	15	27	"	0/10
4	14	16*	"	3/4
5	14	20*	"	2/6
6	12	27	Wild BLU 23	1/10
7	6	21	"	0/10
8	12	27	"	0/10
9	11	21	"	2/10
10	10	24	"	2/10
11	2	38	Lab. [1] BLU 23	0/9
12	2	20	"	0/9
13	2	27	"	0/9
14	2	31	"	0/9
15	3	38	"	0/9
16	3	31	Wild BLU 23	0/9
17	2	24	"	0/9
18	2	27	"	0/9
19	3	24	"	0/9
20	2	27	"	0/9

[1].Laboratory adapted virus
*Bull died

Serotype 1 in mature bulls (5-6 years)

Laboratory Adapted Virus: All 8 bulls became viraemic with the longest duration of viraemia being 27 days (Table 4). Bluetongue virus was identified in a total of 22 semen samples from 5 bulls (1-6 ejaculates). In 2 of these 5 bulls virus was only detected in the semen concurrently with the period of viraemia. Virus was detected in the semen of the other 3 bulls for up to 3 collections over a period of 10 days beyond the period of detectable viraemia. Although semen collections from the 5 bulls continued for a further 12 weeks, no further virus isolations were made.

"Wild" Virus: All 8 young bulls became viraemic with the longest duration of viraemia being 23 days. All semen samples were negative for virus when inoculated into sheep.

Table 4.Detection of BLU 1 in the semen of experimentally infected bulls.

Bull	Age (years)	Duration of viraemia (days)	Virus	Ejaculates with BLU 1/ejaculates tested
A	6	13	Lab. [1] BLU 1	5/20
B	5	20	"	5/20
C	6	23	"	0/12
D	6	13	"	0/12
E	5	16	"	0/12
F	5	27	"	5/12
G	5	16	"	6/20
H	6	27	"	1/12
I	6	17	Wild BLU 1	0/12
J	6	13	"	0/12
K	6	13	"	0/12
L	6	10	"	0/12
M	5	7	"	0/12
N	6	17	"	0/12
O	6	13	"	0/12
P	5	23	"	0/12

[1]Laboratory adapted virus

Serotype 1 in young bulls (less than 2.5 years)

Laboratory Adapted Virus: Each of the 8 young bulls inoculated with laboratory adapted virus became infected, with patterns of viraemia similar to the old bulls. Virus was not identified in the semen of any of these bulls.

"Wild" virus: Each of the 8 young bulls inoculated with "wild" virus became infected, with patterns of viraemia similar to the old bulls. Virus was not identified in the semen of any of these bulls.

(iii)**Latent Infection**

Northern Territory: Six bulls previously infected with either BLU1 or 23 were immunosuppressed with dexamethasone. There was no evidence of a resurgence of viraemia and no evidence of bluetongue viruses in the semen. Bluetongue was not isolated from any tissues of the reproductive tract or spleen when the bulls were slaughtered.

NSW: When 3 of the bulls (A, B and G) which had excreted virus in their semen were treated with corticosteroids there was no evidence of a resurgence of viraemia and there was no further shedding of bluetongue in the semen.

Bluetongue was not isolated from any tissues of the genital tract or spleen when 5 of the bulls were slaughtered 56 days post inoculation and 3 bulls were slaughtered 112 days post inoculation. There were no gross lesions apparent in the tissues of any of the bulls at necropsy. Histology on the tissue sections confirmed the absence of pathological changes.

(iv)**Discussion**

Results of the experimental infection of bulls are summarised in table 5.

Table 5.Bluetongue virus in semen.

	Old Bulls		**Young Bulls**	
	BLU 1	**BLU 23**	**BLU 1**	**BLU 23**
Laboratory adapted virus	5/8	3/5	0/8	0/5
Wild virus	0/8	3/5	0/8	0/5

Serotype 1: These studies show that BLU 1 can be excreted in the semen of old bulls experimentally infected with laboratory-adapted virus but not those infected with "wild" BLU 1.

Overall, 22 semen samples containing BLU 1 were identified from 5 of the 8 bulls infected with laboratory adapted virus. Despite a similar infective dose of "wild" BLU 1

as the inoculum and similar viral titres in the blood, bluetongue was not isolated from the semen of 8 bulls infected with this virus. These results add further weight to the hypothesis that bluetongue rarely occurs in the semen of naturally infected bulls.

The presence of bluetongue in the semen of bulls inoculated with laboratory-adapted serotypes was shown to be intermittent and closely associated with the period of viraemia. Bluetongue was initially detected in the semen on day 10 post inoculation and was detected in the semen of 3 bulls until day 31. It was also demonstrated in this trial that the titres of BLU 1 in the semen were comparable to viral titres in the blood.

Unlike previous studies, BLU 1 was also isolated from semen outside the period of detectable viraemia. Six virus-contaminated semen samples were identified 10-14 days after the cessation of viraemia in 3 of the 5 excreting bulls. Significant titres of BLU 1 in the semen were also identified at this time (2.76 $\log_{10}CEID_{50}$/ml). This occurrence may have been related to the sensitivity of the isolation procedures employed in this study or to the persistence of BLU 1 in the reproductive tract after viraemia.

The duration of viraemia was longest in those bulls inoculated with laboratory-adapted BLU 1. In bulls infected with laboratory adapted virus, viraemia was evident in all bulls on day 10 post inoculation with most of the bulls exhibiting viraemia over a period of 14-24 days. Analogous viraemic periods have also been reported in similar studies investigating the inoculation of bulls with other laboratory adapted serotypes. There were similar titres and duration of viraemia in those bulls excreting BLU 1 and those bulls not excreting BLU 1 in their semen. This result suggests that excretion of BLU 1 in semen is not a result of higher levels of BLU 1 in the blood of shedding bulls.

BLU 1 was also successfully isolated from the blood of all bulls experimentally infected with "wild" virus. Despite inoculation with a similar dose of BLU 1, those bulls receiving "wild" BLU 1 experienced a slightly shorter viraemic period, although the titres of the viraemias were comparable.

Serotype 23: These results show that BLU 23 can be excreted in the semen of old bulls experimentally infected with both laboratory adapted virus and "wild" virus. When young bulls were used no virus was detected in semen, suggesting that age may be the critical factor in determining seminal contamination with virus.

A total of 6 semen samples containing BLU 23 were identified from 3 of the 5 bulls infected with laboratory adapted virus and 5 semen samples were positive from 3 of the 5 bulls infected with "wild" virus. Recovery of BLU 23 from bulls infected with laboratory adapted virus was probably affected by the death of three bulls before completion of the collection period.

Virus was only detected in the semen of infected bulls during the period of viraemia. The maximum period of viraemia found in the experiments was 38 days. There was no significant difference in the length of viraemia seen with laboratory adapted virus or "wild" virus in either young or old bulls.

Following the observation of blood in the semen of these very old bulls, semen collections which appeared visually normal, were also tested for the presence of blood

using a commercial urine test strip (N-Multistix, Ames). Over three collections the presence of blood or haemoglobin was indicated in 6/6, 5/6, and 5/6 samples respectively. These findings would suggest that the presence of blood in the semen may have been the cause of contamination of these semen samples with bluetongue virus.

The severe clinical disease observed in sheep inoculated with blood and semen of bulls infected with laboratory adapted virus suggests reversion to virulence can occur after a single passage of attenuated virus in cattle. This observation was not investigated further due to a decision not to proceed with research into attenuated vaccines.

Latent infection: With the aim of detecting a latent infection and the resurgence of excretion of virus in semen, 3 bulls which had demonstrated regular seminal shedding of BLU 1 were treated with corticosteroids. Despite the bulls appearing stressed following corticosteroid inoculation, there was no further isolation of BLU 1 from the blood or semen samples. A further 6 bulls infected with either BLU 1 or 23 were similarly immunosuppressed. No bluetongue viruses were detected in either blood or semen of these bulls. When all bulls were slaughtered virus was not isolated from the reproductive tract or spleen of any of the bulls. These results support previous work which questions the existence of a latent state of infection with bluetongue viruses.

Natural infection: The excretion of bluetongue viruses in the semen of naturally infected bulls has restricted the export of their semen. In contrast to the results with experimentally infected bulls, there was no evidence of bluetongue virus in the semen of any of the 41 bulls which had been naturally infected. It is possible that the younger age of these bulls may have been a significant factor contributing to the lack of contamination of semen with virus.

Serology: These trials also demonstrated that the cELISA is a more sensitive diagnostic tool for the detection of bluetongue group antibodies. The appearance of detectable bluetongue group antibodies in both bull and sheep sera were usually evident in the cELISA one week prior to the detection of bluetongue group antibodies in the AGID test. The overall number of samples positive for bluetongue group antibodies was also significantly greater in the cELISA compared to the AGID test. This increased sensitivity for the detection of serogroup antibodies of the cELISA compared with the AGID test supports previous reports.

The most distinctive feature between experiments with regard to the development of an immune response was the titre of neutralising antibodies in the serum of those bulls inoculated with "wild" BLU 1. Although neutralising antibodies were initially detected in the serum of the old bulls at the same time (day 17 post inoculation), the titre of antibodies in the serum of those bulls inoculated with "wild" BLU 1 was up to 4 fold higher. The titre of neutralising antibody in the serum of the bulls infected with "wild" virus was also shown to increase rapidly. Neutralising antibody titres of 512 were recorded in the serum of 4 bulls at day 42 post inoculation. Titres of this level were not recorded in the serum of bulls infected with laboratory adapted virus until day 70 post inoculation. The initial detection of neutralising antibody in all experiments, however, is consistent with previous reports and confirms that virus can exist in the presence of circulating antibodies. The higher titres of neutralising antibody in the serum of bulls inoculated with "wild" BLU 1 probably contributed to the shorter periods of viraemia of

these bulls.

5. SUCCESS IN ACHIEVING OBJECTIVES

These series of trials successfully enabled the relationship between viraemia and shedding of virus in semen to be established. They also confirmed that except in the immediate post viraemic period seropositive bulls did not excrete virus or carry a latent infection after the cessation of viraemia. The importance of the age of the bull and the use of laboratory adapted virus under experimental conditions were clearly identified as factors in previously reported work relating to excretion of virus in semen. While these trials did not completely eliminate the possibility of natural infection of bulls resulting in virus contamination of semen, they did show that this is a rare occurrence restricted to aged bulls or those that have blood contamination of semen.

This information allowed a practical protocol to be devised for the collection of semen from seropositive bulls.

6.PROGRESS IN COMMERCIALISATION.

Not applicable.

7. IMPACT ON MEAT AND LIVESTOCK INDUSTRY

The information is now available to allow a protocol for export of semen from seropositive bulls to be developed. The adoption of this protocol by overseas countries should increase the artificial breeding trade.

8.TOTAL FUNDING

As per original application and contract.

9.CONCLUSIONS AND RECOMMENDATIONS

In summary, it would appear that contamination of bovine semen with bluetongue virus following natural infection is a relatively uncommon occurrence and could be related to contamination of the semen of older bulls with blood.

When live virus vaccines are used for bluetongue, there is a risk of insect transmission of vaccine virus and there may be a short time during, or soon after, the period of viraemia when virus may be found in semen. In Australia, however, in the absence of vaccines for bluetongue, there appears to be minimal risk of the excretion of virus in bull semen. Also, there is no evidence to suggest that semen is contaminated with bluetongue virus at any time other than immediately around the period of viraemia, in this study a maximum period of 6 weeks after infection, or that a latent infection state exists in seropositive bulls. As it is difficult to define the precise time of infection of bulls in the bluetongue endemic areas, especially if animals have already been infected with one serotype and are seropositive, it may be necessary to define a longer qualifying

period before bulls are eligible to provide semen for export. In practical terms it will be necessary to move bulls to a vector free region, hold them for a minimum of 3 months and then it will be absolutely safe to collect semen for export to bluetongue-free countries. Although most countries will accept semen from bulls which are seronegative, sometimes there are stringent additional requirements for confirmatory virus isolation on semen if the bull originates from a vector region. The previously described quarantine conditions would be applicable to bulls regardless of their serological status for bluetongue and will allow unrestricted export of bovine semen without the need for expensive testing of both blood and semen.

The proposed quarantine conditions have been discussed on several occasions with AQIS personnel. It is encouraging to report that new protocols are currently being drafted for the importation of bovine semen from the USA, initially from seronegative bulls at any time of the year and eventually also from seropositive bulls. With a significant relaxation of conditions for importation into Australia, more rational conditions for the export of semen from Australia can be expected. Nevertheless every opportunity should be taken to promote the significant outcome of this project to ensure maximum benefit for industry. It is particularly important that the conservative EEC countries become aware of and accept these results and the logical export certification requirements that would follow. Fortunately, there have been recent opportunities in this direction. Representatives of the Veterinary Commission of the EEC visited Australia during 1994 to discuss progress with disease monitoring programs, recent research results and to inspect laboratory facilities. During this visit Dr Kirkland was able to present at some length the results of this project to the Commissioners who responded very positively.

10.PUBLICATIONS

Melville, L.F., Kirkland, P.D., Hunt, N.T. and Williams, C.F. (1992). Excretion of Bluetongue virus serotypes 1 and 23 in semen of bulls. In "Arbovirus research in Australia". (ed. M.F. Uren and B.H. Kay), Proc 6th Sym: 185-187.