Development of Bluetongue Virus Multicomponent Vaccines Using a Novel Baculovirus System

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Abstract

The difficulties associated with obtaining completely safe, live virus vaccines by the traditional procedures of virus passage and selection of attenuated forms, as well as the risks and expenses involved in preparing killed or subunit vaccines from virulent viruses, may now be overcome using recombinant DNA technologies. Genetic engineering offers a variety of ways of preparing viral vaccines using expression vector systems. Using baculovirus multiple gene expression vectors based on *Autographa californica* nuclear polyhedrosis virus (AcNPV) has provided a new strategy for vaccine development. Such vectors are capable of the simultaneous expression of several foreign genes within the host insect cell in which AcNPV replicates. Using this technology, we have developed multicomponent virus-like particles (VLPs) as virus vaccines and used them as vaccine delivery systems for multiple immunogens. The VLP technology is a completely new technical development from a novel baculovirus expression vector system, which has the capability of simultaneously co-expressing up to five proteins in insect cell culture. The transformed cells are stable, and authentic protein product is produced at high levels. This novel technology has been used for the production of pilot-scale quantities of a recombinant bluetongue virus (BLU) multi-subunit vaccine (VLPs immunologically indistinguishable from BLU). Preliminary clinical trials have verified this vaccine's safety and efficacy. Unlike live virus vaccines, VLPs are non-infectious and lack virus (or other) DNA/RNA required for replication. VLPs do not replicate in host cells. However, trials in sheep have shown that VLPs are more immunogenic than subunit vaccines (viral proteins) or than viruses inactivated chemically. In addition, VLPs are effective at eliciting humoral, cell-mediated and mucosal immunities, and are safe to produce and handle. (As the baculovirus vector and host cells used to make VLPs do not come from mammalian sources, they do not contain mammalian derived pathogens.) The expression system described in this paper is a tool which may have a range of applications in industries employing biotechnology to produce vaccines, insecticides, or diagnostic and protein reagents. Diagnostic reagents developed for BLU and African horsesickness virus are discussed as examples.

GENETIC engineering offers a variety of approaches for viral vaccines. An exciting advance in this field is the ability to construct virus-like particles (VLPs) which resemble their natural counterparts but lack genetic information (i.e. are unable to replicate). We have developed VLPs based on bluetongue virus (BLU), the causative agent of bluetongue disease of sheep.

To date, 24 BLU serotypes (BLU1, BLU2, etc.) have been identified from different parts of the world (Erasmus 1990). In the past, live attenuated orbiviruses have been used as reasonably effective vaccines in those regions where bluetongue virus causes epidemics of disease in livestock. However, as the BLU genome comprises 10 double-stranded (ds) RNA species, there is some concern that using live attenuated viruses in vaccines may maintain the virus in the wild, and facilitate RNA segment reas-

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sortment and the generation of new viral genotypes. This is especially important as not all the attenuated derivatives of the known BLU serotypes appear adequately attenuated.

The Bluetongue Virus

The architecturally complex, 810 Å diameter bluetongue virus can attach to, and replicate in, a variety of vertebrate cells (Roy, 1996). The virion contains seven structural proteins, four of which are organised into two protein shells. The outer shell contains two major protein species, VP2 (110 kD) and VP5 (58 kD), while the inner shell has two other major species, VP3 (100 kD) and VP7 (38 kD). The icosahedral inner shell encapsidates the virus genome and three other (minor) proteins; VP1 (150 kD), VP4 (76 kD) and VP6 (36 kD). In addition, three or four non-structural proteins are synthesised in virus-infected cells. Of the ten proteins, only VP2 and VP5 vary from serotype to serotype. All five core proteins and the three non-structural proteins are highly conserved (Roy et al. 1990a). Each protein is encoded by a single BLU dsRNA segment (Mertens et al. 1984). To develop rationally-designed BLU vaccines in the past few years, we have been developing an understanding of the structural and functional relationships of BLU genes and gene products, and of the assembly of the gene products for the formation of virions.

Baculovirus Expression Vectors

Recent advances in gene manipulation have made it possible to express foreign genes in heterologous systems. The productivity and flexibility of insect baculovirus expression vectors, and the ability of the baculovirus genome to incorporate (and express) large amounts of foreign DNA in *Spodoptera frugiperda* insect cells, have permitted this system to be used for the expression not only of a single gene, but also for the simultaneous expression of dual and multiple genes. To accomplish this, several expression vectors have been developed based on the resident promoters of AcNPV, the nuclear polyhedrosis virus of *Autographa californica* (Bishop 1992; Belyaev and Roy 1993; Belyaev et al. 1995).

Using these various expression vectors, we have expressed all 10 BLU genes, either individually or in various combinations, using single, dual, triple and quadruple expression vectors, and have analysed the structure-function of each gene and gene product (Roy 1992). However, only the structural characteristics and functional attributes of those proteins relevant to generation of vaccines are discussed in this paper.

Assembling BLU Core-like Structures with Baculovirus Expression Systems

Core-like particles

The flexibility of baculovirus expression vectors and the capacity of the baculovirus genome to accommodate large amounts of foreign DNA has allowed us to exploit the system for the simultaneous expression of multiple BLU genes in a single insect cell. Our initial effort was to assemble the two major core proteins, VP3 and VP7. For this purpose, a dual baculovirus expression vector consisting of duplicated polyhedron promoters (PH) of AcNPV, with downstream transcription terminator sequences, was used to express the coding sequences of the L3 (VP3) and S7 (VP7) genes of BLU (French and Roy 1990). Recombinant baculoviruses synthesising both proteins were isolated and produced core-like particles (CLPs) distributed throughout the infected insect cells. Gradient-purified CLPs were similar in size and appearance to cores prepared from BLU (Fig. 1). Only VP3 and VP7 were identified as the protein components of the expressed particles and the molar ratios of these two proteins were similar to those of VP3 and VP7 derived from infectious BLU. The CLPs appeared to lack nucleic acids when analysed by phenol-chloroform extraction and alcohol precipitation.

Three-dimensional structures of BLU cores and CLPs

To determine whether CLPs mimic the morphology of BLU-derived core particles, cryoelectron microscopy (cryo-EM) was used to examine unstained, unfixed virus-derived core particles (i.e. avoiding heavy metal stains, fixture and dehydration: Prasad et al. 1992; Hewat et al. 1992a). Using image reconstruction methods, the micrographs of cores at 30 Å resolution revealed that the particles were 690 Å in diameter, exhibited an icosahedral symmetry and contained surface knobs organised with a triangular number of 13 (Prasad et al. 1992). The surface consisted of clusters of VP7 trimers providing 260 prominent knob-like protrusions (780 VP7 molecules) organised into pentameric and hexameric units with channels in between (Fig. 2). A total of 132 channels per particle were identified, involving all three-fold axes. The aqueous channels are approximately 70 Å deep and 80 Å wide at the surface. Some channels penetrate to the inner layer, and are probably the pathways for metabolites to reach the sites of viral mRNA transcription and the export of nascent mRNA molecules out of the cores. The underlying smooth scaffold for the VP7 trimers consists of the second major core protein, VP3, the organisation of which was not fully revealed at 30Å resolution (Fig. 2). The VP7 and VP3 enclose the inner core, which comprises the three

minor proteins VP1, VP4 and VP6 and the genomic dsRNA. We do not yet know how these are organised with respect to one another or to VP3 and VP7.

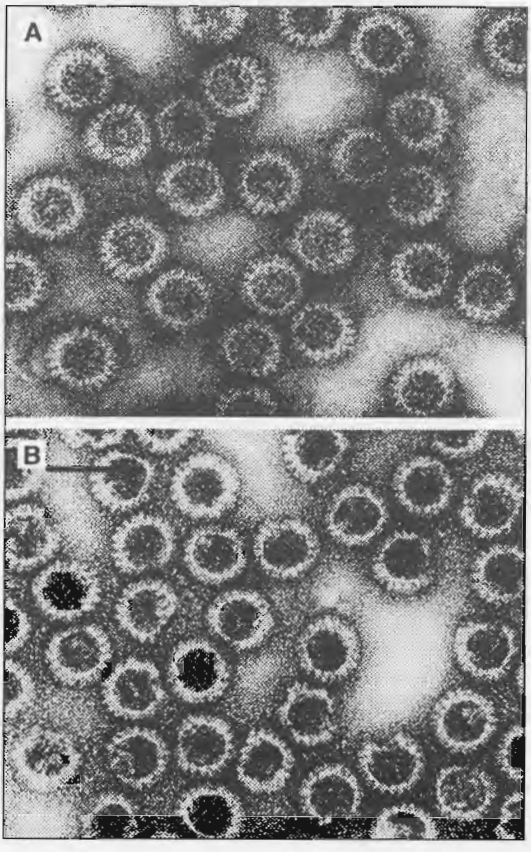

Figure 1. Electron micrographs of (A) authentic BLU cores and (B) baculovirus-expressed core-like particles (CLPs).

The images of bacułovirus-synthesised CLPs at relatively low resolution (65 Å) revealed a similar icosahedral configuration (T=13 for the surface layer) and an identical diameter (690 Å) to that of BLU cores. Some of the synthetic CLPs lacked the full complement of VP7. In these, the shapes of the VP7 trimers were clearer (Hewat et al. 1992a). The trimers have tripod-like shapes and each consists of an upper (outermost) and lower (innermost) domain. The shapes and structures of VP7 trimers have recently been confirmed by X-ray crystallographic data (Grimes et al. 1995). It appears that the VP3 molecules are roughly disc-shaped and that dimers of VP3 form the building blocks for the icosahedral structure. There are a total of 120 VP3 molecules per virion, organised in a triangulation number of T=1. VP3 subcores have been purified from CLPs. The cryo-EM data obtained for purified subcores appear similar to those deduced from VP3 in CLPs (Hewat et al. 1992a).

Incorporation of the three minor proteins within CLPs

To determine whether the three minor proteins can be assembled into CLPs, VP1 and/or VP4 and/or VP6 have been co-expressed using baculovirus vectors together with VP3 and VP7. VP1, the putative viral polymerase, was readily incorporated within CLPs. When the VP7 trimers were removed, the derived subcores consisted only of VP3 and VP1, demonstrating that VP1 interacts with VP3 (Loudon and Roy 1991). Similar results were obtained with VP4 and VP6, and for combinations of all three minor proteins (Le Blois et al. 1991). However, unlike VP1 or VP4, VP6 was only poorly incorporated into CLPs. Since VP6 is a highly basic protein and readily associates with RNA (single- or double-stranded), VP6 may chaperone the incorporation of RNA into particles (or vice versa) and may only be poorly incorporated in the absence of RNA (Roy et al. 1990b; Hayama and Li 1994).

Various assay systems have been developed using purified CLPs and single-stranded RNA species synthesised in vitro to determine whether CLPs without the minor protein components retain the ability to interact with viral RNA species. The data indicate that the RNA-binding affinity of CLPs involves VP3 but probably not VP7 (Loudon and Roy 1992). How RNA interacts with VP3 is unknown.

Assembly of bluetongue virus-like particles (VLPs) using baculovirus vectors

Baculovirus multigene vectors have been developed to co-synthesise up to five BLU proteins in the same cell (Belyaev and Roy 1993; Belyaev et al. 1995). In addition to the PH promoter, copies of the p10 promoter of AcNPV have been utilised to facilitate the high level co-expression of several proteins in each infected cell. For optimum synthesis of VLPs, a quadruple gene expression vector has been used to synthesise BLU VP2, VP3, VP5 and VP7 proteins. The expressed proteins assembled into virtually homogenous double-capsid particles (Fig. 3). Co-infections with single or dual gene expression vectors gave VLPs that contained different amounts of the outer capsid proteins, depending on the experiment (French et al. 1990). The formation of complete VLPs in the absence of the non-structural proteins implies that the latter are not necessary for the assembly of double-capsid particles (or CLPs). VLPs express high levels of hemagglutination activity, similar to that of BLU virions. Antibodies raised to the expressed particles gave high titres of neutralising activity against the homologous BLU serotype (French et al. 1990).

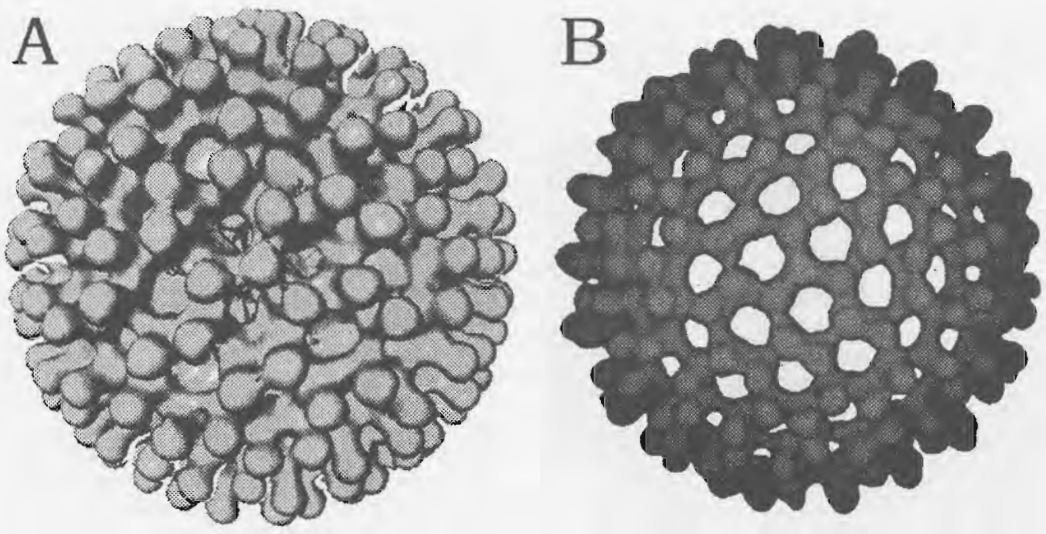

Figure 2. Surface representation of the cryoelectron micrographs of the BLU core viewed along the icosahedral three-fold axis, (A) showing the knob-like protrusions of VP7 trimers in the outer layer and (B) the density in the outer layer showing the large holes or channels at all the five- and six-coordinated positions formed by the arrangement of the VP7 trimers.

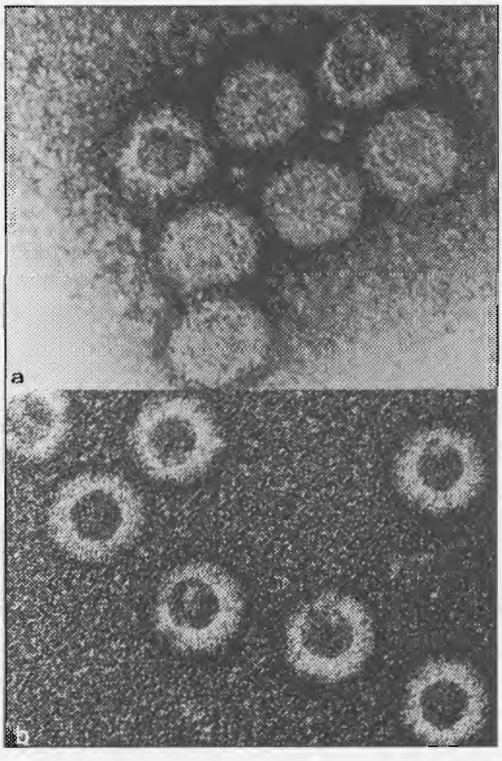

Figure 3. Electron micrographs of (A) BLU virions, and (B) baculovirus-expressed double-capsid virus-like particles.

The three dimensional structures of BLU virions and VLPs

The three dimensional structure of BLU has been determined using image analysis of cryo-electron micrographs of virion particles (Hewat et al. 1992b). The reconstruction revealed a morphology contrasting sharply with that deduced by conventional negative-staining methods. The outer capsid exhibits an icosahedral configuration (860 Å in diameter) and a well-ordered morphology (Fig. 4). The two proteins of the outer capsid have distinctive shapes; one globular and almost spherical, the other sail-shaped. The 120 globular proteins sit neatly on each of the six-membered rings of the VP7 trimers of the core. The sail-shaped spikes are situated above 180 of the 260 VP7 trimers and form 60 triskelion-type motifs which cover all but 20 of the VP7 trimers. These spikes are probably the VP2 hemagglutinating and neutralisation antigens. The two proteins appear to form a continuous layer around the core, except for holes on the five-fold axis. Three dimensional reconstruction of VLPs at 55 Å resolution is comparable to that of authentic virions with diameters of 860 Å, and exhibiting essentially the same basic features and full complement of the four proteins (Fig. 4; Hewat et al. 1994).

Assembly of Heterologous VLPs Using Different BLU Serotypes

Twenty-four BLU serotypes are recognised. The outer capsid proteins (VP2 and VP5) exhibit the least

conservation among BLU serotypes, in contrast to all the other virus-coded proteins (Roy et al. 1990a). VP2, the main serotype-specific antigen, is the most variable: VP2 sequence identity comparisons between BLU1, 2, 10, 11, 13 and 17 range from 39–73%. Antisera raised to the baculovirus-expressed VP2 of particular BLU serotypes neutralise the homologous virus and, depending on the antigen, to some extent cross-neutralise certain other BLU serotypes (Inumaru and Roy 1987; Urakawa et al. 1994). These data indicate that some BLU serotypes are more closely related than others. In contrast to VP2, the primary sequences of the VP5 proteins of some BLU serotypes are more similar, sharing up to 94% identical amino acids (Hirasawa and Roy 1990; Oldfield et al. 1991; Yang and Li 1992).

The structural compatibilities of various VP2 and VP5 species in VLP formation have been investigated. Heterologous VLPs have been sought by co-infection of insect cells with appropriate recombinant baculoviruses (Loudon et al. 1991). Assembled particles were purified and analysed by electron microscopy and SDS-PAGE to confirm their authenticity. The presence of VP2 and VP5 on VLPs was demonstrated by hemagglutination and Western immunoblotting respectively. Despite the high level of sequence variation amongst the different serotypes, the VP2 and VP5 proteins of six different BLU serotypes formed VLPs with the VP3 and VP7 of another source (Loudon et al. 1991). Some combinations of VP2 and VP5, such as the VP2 of BLU11 or BLU17 and the VP5 of BLU10, resulted in the assembly of particles, while other combinations, such as the VP2 of BLU2 and the VP5 of BLU10, did not (Table 1). The data indicate that VP2 may require the VP5 of the same or a closely related serotype (e.g. BLU10 or BLU17) but may not form VLPs efficiently with the VP5 proteins of more distant serotypes.

VLPs as Vaccines

Since recombinant baculovirus-derived VLPs elicited strong neutralising antibodies in guinea pigs, it can be anticipated that, in sheep, VLPs should elicit protective responses against bluetongue viral infection. Several experiments were performed to examine the protective efficacy of VLPs in sheep. Each experiment used groups of BLU-free, BLU-susceptible, one-year-old Merino sheep kept in an insect-proof isolation stable. In the first experiment (Table 2), one group of eight sheep (two sheep for each concentration of protein) was immunised subcutaneously with 2 mL unpurified VP2, or VP2 and VP5 proteins, or VLPs in saline, containing known amounts of protein and suspended in 50% Montanide Incomplete Seppic Adjuvant (ISA-50, Seppic, Paris). As a control, one group of sheep received only saline.

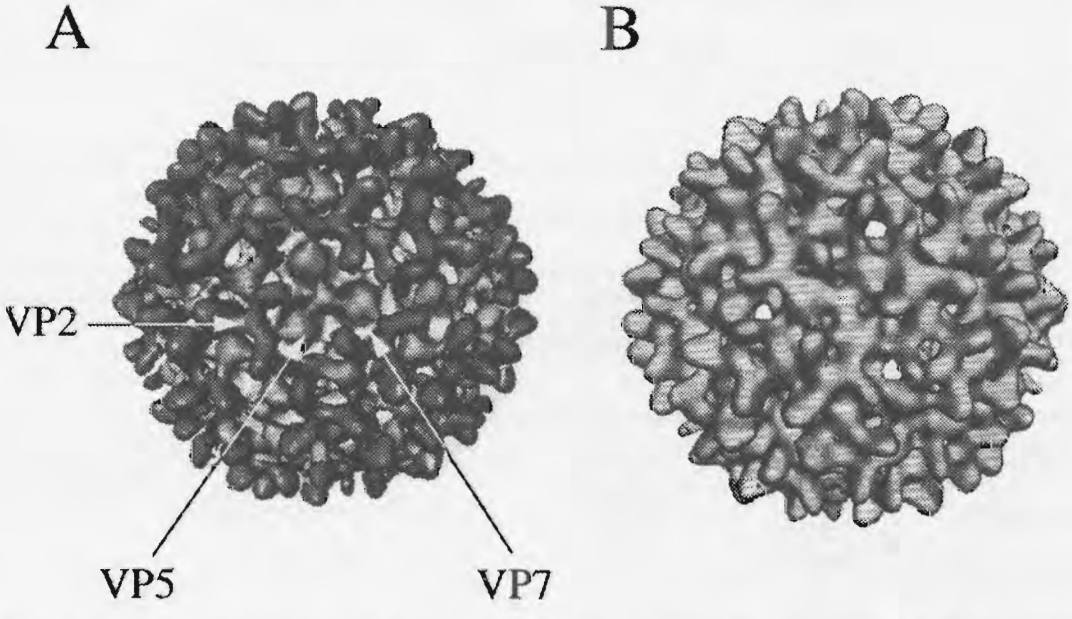

Figure 4. Surface representation of a cryoelectron micrograph of (A) an icosahedral virus particle and (B) VLP, viewed along a two-fold axis, showing the topography of the two outercapsid proteins, one globular-shaped (VP5) and the other sail-shaped (VP2). VP7 trimers, underneath, are indicated.

Each immunised animal was boosted with the same amounts of protein on day 21 post-immunisation. From day 21 to the day of challenge, serum from each animal was collected at intervals (Table 2) for virus neutralisation tests. Antibody titres were expressed as the reciprocal of the serum dilution estimated to cause a 50% reduction in plaques. As summarised in Table 1, sheep receiving VLPs developed demonstrable neutralising antibodies, albeit to different levels (Roy et al. 1992, 1994a). The levels of neutralising antibodies depended on the amount of VLPs administered. Significant levels of neutralising antibodies were elicited with all concentrations of VLPs and persisted throughout the study. The control sheep inoculated with saline remained seronegative. All sheep were challenged by subcutaneous inoculation of 1 mL of infective sheep blood containing virulent BLU10 (SA strain) either at day 75 or 117 (Table 2). The animals' clinical reaction indices (CRI) and viraemia were monitored from days 3 to 14 post-challenge (Huismans et al. 1987). Antibody titres of serum collected up to 21 days were also monitored. The challenged sheep developed neither clinical signs nor viraemias, indicating suppressed replication of bluetongue virus. The post-challenge blood samples of the sheep that received only saline were viraemic: these sheep developed high neutralising antibody titres indicative of a primary infection. To summarise this experiment, protective immunity to bluetongue disease was obtained by vaccinating sheep with doses of 10 µg or more of BLU VLPs as well as with high doses of outercapsid protein VP2 or VP2 and VP5.

To analyse further the protective effects and duration of VLP vaccination, a similar protocol was employed in a second experiment for VLPs (10 µg or 50 µg per sheep) representing BLU10 and BLU17 (Roy et al. 1994a). The neutralising antibody titres of the vaccinated sheep were determined at weekly intervals and during a 60-week period after the booster. Both types of VLP elicited antibodies (to various levels) that neutralised the homologous virus. In almost all cases these neutralising titres remained high throughout the 60-week period. The neutralising antibody titres for the animals receiving 50 µg doses of VLPs were not significantly higher than those in animals receiving 10 µg doses (Table 2). Sheep vaccinated with a mixture of the two types of VLPs developed antibodies that neutralised both types of virus as well as some related heterologous viruses (e.g. BLU4) when tested by plaque reduction assays. As expected, the control sheep inoculated with saline remained seronegative.

All sheep were challenged 14 months after the booster vaccination by subcutaneous injection of vir-

Table 1. Formation of VLPs across different bluetongue serotypes.

	VP2 origin					
VP5 origin	BLU1	BLU2	BLU10	BLU11	BLU13	BLU17
BLU2	+	+	–	–	–	–
BLU10	–	–	+	+	–	+
BLU13	nd	nd	–	–	+	–

Notes:

+ and – indicate presence and absence respectively of double-shelled virus-like particles.
nd = experiment not yet done.

S. frugiperda cells were infected with combinations of the following recombinant baculoviruses: a dual recombinant expressing VP3 and VP7; a single recombinant expressing VP2 of serotypes 1, 2, 10, 13 or 17; and an additional single recombinant expressing the VP5 of serotypes 2, 10, or 13. The cells were lysed three days post-infection and the resultant particles isolated on discontinuous sucrose gradients. Purified particles were examined under the electron microscope.

Table 2. Protective efficacy in sheep of BLU10 outer capsid proteins and VLPs against bluetongue.

Inoculum	No. of sheep per experiment	Neutralising antibodies	Time of challenge	Clinical Reaction Index (CRI)	Viraemia (days post challenge)
VP2 (100 µg)	4–8/group	4–256	75 days	0	0
VP2 (50 µg) plus VP5 (20 µg)	4–8/group	4–256	75 days	0	0
VLPs (10 or 50 µg)	4–8/group	4–256	4 or 14 months	0	0
Saline control	4/group	0	75 days to 14 months	3.6–9.7	D4–14

ulent virus. Animals challenged with homologous viruses (BLU10, BLU17) were completely protected and showed no clinical reactions: this included animals receiving 10 μg VLP doses (Table 3). No viraemias were detected in these animals after challenge. Some animals with 50 μg VLP doses were also protected to a low level when challenged with heterologous virus. In comparison, control animals developed viraemias with high or moderate signs of disease (BLU10 gave CRI of 7.1–8.0; BLU17 gave CRI of 1.6–2.7). Summarising this experiment, the data showed that vaccination with VLPs provided long-lasting protection against homologous BLU challenge. There was some preliminary evidence of cross-protection, depending on the challenge virus and the amounts of antigen used for vaccination (Roy et al. 1994a).

Table 3. Protection of sheep by BLU VLPs against heterologous BLU challenge.

Immunised with BLU10, or BLU17, or BLU10 + BLU17 VLPs
(10 or 50 μg/sheep), 4–8 sheep per group
Pre-challenge antibodies: 4–256
Post-challenge (14 months) with BLU4, 10, or BLU17
Clinical reactions — none (BLU4, 10, 17)
Viraemias — none (BLU4, 10, 17)

Protection Afforded by Cores

We investigated whether CLPs containing the two conserved proteins VP3 and VP7 would provide any homologous and heterologous protection against BLU by a cell mediated mechanism. For the initial studies, we used two groups of five sheep each. One group was inoculated with 50 μg BLU10 CLP in ISA50. The second group was boosted on day 21 and challenged with BLU10 two weeks later.

All post-challenge sheep developed viraemias and neutralising antibodies (Table 4; Roy et al. 1994b). However, with the exception of fever, the vaccinated sheep developed only slight clinical reactions while controls showed characteristic mouth and feet lesions is well as fever. The vaccinated sheep had an average CRI of 3.5 compared to an average of 9.0 for the control sheep. Thus CLP vaccination provided partial protection against BLU challenge. Further experiments are planned to study this aspect further.

Conclusion

Our results have clearly demonstrated that VLPs are highly immunogenic even at low doses. The sheep given 10 μg doses of VLPs actually received 1–2 μg of VP2 (10–20% of the VLP mass). This compares well with the much higher amounts required for success in protection studies with VP2 alone, or with VP2 and VP5 mixtures (Roy et al. 1990c). There are several possible explanations. First, the conformational presentations of the relevant epitopes on VP2 probably mimic those on the authentic virus. Second, both VP2 and VP5 are present. Third, the VP3 and VP7 may provide a necessary scaffold for VP2 and VP5 antigen presentation. Fourth, any of the four BLU proteins may have a direct role in eliciting cell-mediated immunity induced by the BLU VLPs. These results suggest that this technology has much to offer for development of vaccines for both veterinary and human diseases. Moreover, there is every reason to believe that it should be possible to make vaccine chimeras representing different BLU serotypes, for example involving the expression of several BLU VP2 genes.

Table 4. Protective efficacy in sheep of BLU core-like particles (CLPs) against bluetongue.

Inoculum	Sheep no.	Neutralising antibody	Clinical Reaction Index (CRI)
CLPs	1	320	1.5
	2	160	5.6
	3	320	3.8
	4	160	2.7
	5	160	4.0
Saline control	6	320	11.4
	7	320	9.5
	8	320	12.3
	9	320	6.8
	10	160	5.1

Note:
Immunised and control sheep developed viraemias at high neutralising antibody titres. However, clinical signs of bluetongue were less in the vaccinated animals (CRI 1.5–5.6, average 3.5) compared to the controls (CRI 5.1–12.3, average 9.0). Only controls showed characteristic mouth and feet lesions.

Currently, we are also developing BLU CLPs and VLPs to deliver multiple peptide components representing other viral epitopes, to elicit protective immunity agents for different viral diseases. As BLU CLPs and VLPs are large multiprotein structures, they can incorporate alternative protein forms of the structural proteins (e.g. chimeric VP7), including alternative forms of these proteins (eg. VP7a, VP7b, etc.). It may be possible to use more than one protein type to deliver antigens (eg. VP2, VP5, VP7). Moreover, VLPs based on alternative BLU serotypes can be used

for successive immunisations to evade the anti-BLU response elicited in a primary vaccination. This is another novel feature of this system not currently available in other antigen delivery systems.

The CLPs and VLPs of bluetongue viruses offer several particular advantages over other systems.

- Large quantities can be produced due to the expression capabilities of baculovirus vectors (ca. 20–30 mg per litre of culture, produced in serum-free medium, stable to freeze-drying, etc.).
- CLPs and VLPs can be purified using a one-step generic protocol based on the physical properties of the particle (gradient centrifugation of cell lysates).
- They are devoid of any detectable amount of insect or baculovirus proteins, RNAs, or DNAs.
- The purification procedure is gentle enough to maintain the morphological structure of the particles in their native conformations.

  The particles cannot replicate although multiple epitopes could be accommodated.
- Most importantly, VLPs have inherent properties of inducing both B cell and T cell responses in vertebrate hosts.

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Bluetongue Recombinant Vaccines

S.J. Johnson* and P. Roy†

Abstract

Bluetongue virus-like particles (VLPs) and core-like particles (CLPs) were administered to Merino sheep to assess the potential of the particles as a vaccine against bluetongue. Sheep given 2 doses of 10 μg VLPs at a 35-day interval produced group and homologous neutralising antibodies. Two weeks after the second dose the sheep were resistant to challenge with homologous virus but showed little resistance to challenge with virulent heterologous virus. Sheep inoculated twice at a 35-day interval with 10 μg CLPs showed strong group antibody response but no neutralising antibodies. These sheep were resistant to challenge with both homologous and heterologous virus when challenged 14 days after the second dose.

FOLLOWING the initial discovery of bluetongue virus (BLU) in Australia (St. George et al. 1978) and the subsequent identification of additional serotypes (St. George et al. 1980; Gard et al. 1985, 1987a, b), some Australian serotypes were found to be pathogenic for Merino sheep (Johnson et al. 1989; Melville and Gard 1990).

Attenuated vaccines being developed in Australia were teratogenic (Johnson et al. 1991; Flanagan and Johnson 1996). Production of non-replicating recombinant double-shelled virus-like particles of BLU (French et al. 1990), lacking nucleic acid, resulted in the correct spatial arrangement of BLU antigenic proteins necessary for successful vaccines. Preliminary vaccine studies using recombinant proteins in South Africa showed promising results (Roy et al. 1990, 1992) and the particles were imported into Australia to assess their efficacy against Australian BLU serotypes in Merino sheep.

Materials and Methods

Animals

Three-year-old Peppin Merino wethers from the Queensland Department of Primary Industry's Toorak Research Station were used in all trials. All sheep tested seronegative to BLU by enzyme linked immunosorbent assay (ELISA). Animals were housed in an isolation unit where food and water were available ad lib.

Regimen

Sheep were vaccinated twice (on day 0 then on day 21 or 35) with 10 μg recombinant proteins. An equal volume of Incomplete Seppic adjuvant 50 was used on each occasion. Control sheep received only adjuvant. From all sheep, 10 mL of blood was taken every seven days before challenge and daily for 18 days after challenge.

Challenge viruses had been recovered from sentinel cattle in Northern Territory and passaged three times in sheep only. Each sheep received 10 mL (5 mL intravenously, 5 mL subcutaneously) of infected blood containing $10^{4.0}$ $TCID_{50}$ virus per mL. After challenge sheep were examined clinically and temperatures recorded daily and haematological values measured every second day.

Postchallenge viraemia was estimated by inoculating serial 10-fold dilutions (10–2 to 10–7) of sonicated blood into embryonated chicken embryos (ECE), using six embryos per dilution. The numbers of embryos dying per dilution on days 3 to 7 postinoculation were used to calculate the 50% chicken embryo lethal dose end points by the method of Reed and Muench (1938). Serology was carried out by standard methods (St. George 1981).

Results Virus-like Particles

Forty sheep were used (20 vaccinates, 20 controls). VLPs contained VP2, VP5, VP3 and VP7 of Austral-

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ian BLU1. Vaccinations were given at a 35-day interval: on day 77 after secondary vaccination half of each group was challenged with Australian BLU1 and half with Australian BLU23.

Serology

Only 13 sheep (65%) reacted in ELISA after primary vaccination, but all sheep were positive by day 28 after secondary vaccination. No neutralising antibodies were detected in any sheep after primary vaccination. Ten sheep (50%) had detectable neutralising titres by day 28 after secondary vaccination and 12 (60%) by day 47 after secondary vaccination (Table 1).

Temperature

The temperature response of vaccinates was significantly less than that of controls ($P < 0.01$) following challenge with BLU1, but there was no significant difference in temperature response after challenge with BLU23 (Fig. 1).

Haematology

Total leucocyte counts were lower in controls than in vaccinates for both challenge groups (Fig. 2).

Table 1. Serological status of sheep vaccinated with baculovirus-expressed recombinant bluetongue virus-like particles on days after primary and secondary vaccination.

Sheep no.	Days after secondary vaccination																	
	d 21 APV		7			28			34			47			77			
	A	E	A	E	S	A	E	S	A	E	S	A	E	S	A	E	S	
915	+	–	++	38	10	+++	63	5	++	73	5	+	33	60	+	39	160	
916	+	–	+++	51	–	+++	50	–	+++	60	–	+++	52	–	+++	53	–	
617	++	–	+++	49	–	+++	45	–	++	42	–	+	40	–	+	67	–	
920	+	–	+++	32	–	+++	45	10	++	57	–	+	44	10	+	46	10	
921	–	–	+++	28	–	+++	47	–	+++	57	–	+++	45	–	+++	32	–	
922	+++	12	+++	42	–	+++	41	–	+++	43	–	+++	43	–	+++	51	–	
924	+	–	+++	52	10	+++	60	5	+++	60	5	++	43	10	+++	47	10	
926	+	2	+++	48	10	++	47	–	++	46	–	+	46	–	++	57	–	
927	–	–	+++	40	–	+++	56	–	++	52	–	++	52	–	++	54	–	
928	+++	37	+++	53	–	+++	60	–	+++	44	–	+++	48	–	+++	61	–	
929	–	–	++	35	–	+++	42	–	++	42	–	+	40	–	++	51	–	
931	+++	27	+++	52	–	+++	48	–	+++	51	–	+++	46	40	+++	53	10	
938	+	–	+++	50	–	+++	53	5	++	50	5	NS	NS	30	++	46	40	
945	+	–	+++	38	15	+++	44	30	+	40	10	+	48	60	+	46	40	
947	–	–	–	37	–	–	58	–	–	38	–	–	28	10	–	20	–	
950	–	–	+++	37	5	+++	45	10	+	61	5	+	48	60	+	52	60	
951	–	–	–	–	–	++	36	5	+	48	5	+	55	40	+	40	5	
954	–	–	+++	49	10	+++	40	5	++	42	5	+++	46	160	+++	53	120	
955	+++	16	+++	32	10	+++	53	5	++	56	5	+++	43	120	++	54	160	
960	–	–	+++	64	5	+++	50	5	+++	52	5	+++	52	120	+++	53	120	

A agar gel immunodiffusion test
E ELISA
S serum neutralisation test
APV after primary vaccination
ASV after secondary vaccination
NS no sample

Clinical signs

Vaccinated sheep showed no clinical signs of bluetongue after challenge with BLU1. Controls challenged with BLU1 showed mild hyperaemia of buccal and nasal mucosa and transient lameness. All sheep challenged with BLU23 showed moderate hyperaemia of buccal and nasal mucosa, serous nasal discharge, lameness, mild facial and subventral oedema, and transient dyspnoea (Figs. 3, 4, 5, 6 and 11). One (10%) control sheep died of severe bluetongue disease on day 17 postchallenge.

Postchallenge viraemia

Postchallenge viraemia was significantly lower ($P < 0.01$) in vaccinates than in controls after day 6 postchallenge for sheep challenged with BLU1, although there was no significant difference in level of postchallenge viraemia between vaccinates and controls challenged with BLU23 (Fig. 7).

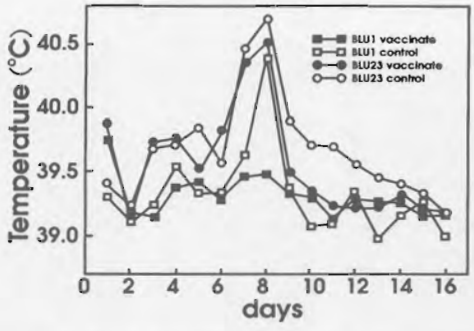

Figure 1. Group mean temperatures after challenge with Australian BLU1 and BLU23 in sheep vaccinated with baculovirus-expressed recombinant BLU virus-like particles.

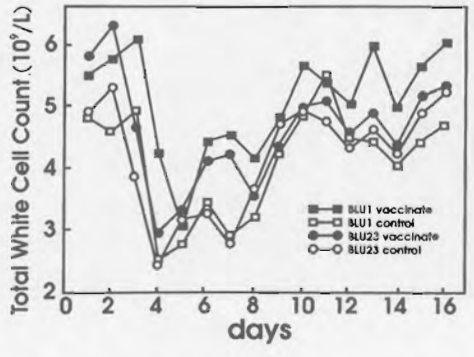

Figure 2. Group mean total leucocyte counts after challenge with Australian BLU1 and BLU23 in sheep vaccinated with baculovirus-expressed BLU virus-like particles.

Figure 3. Head of a Merino sheep infected with BLU23 showing severe oedema.

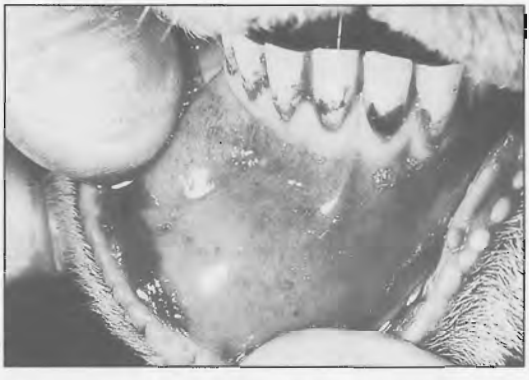

Figure 4. Buccal cavity of a Merino sheep infected with BLU23 showing hyperaemia and haemorrhage.

Figure 5. Thorax of Merino sheep infected with BLU23 showing hydrothorax.

Figure 6. Thorax of a sheep which died from BLU23 infection, showing haemorrhages in the wall of the aorta.

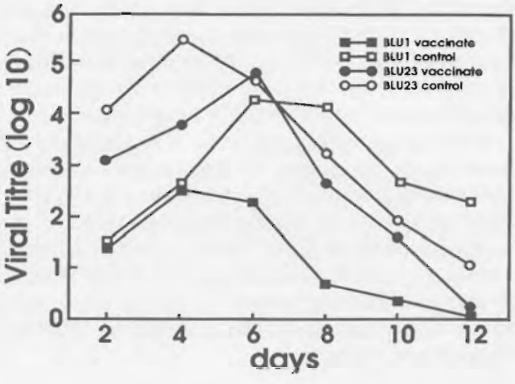

Figure 7. Group mean viraemia following challenge with Australian BLU1 and BLU23 in sheep vaccinated with baculovirus-expressed recombinant BLU-like particles.

Results Core-like Particles

Thirty sheep were used (20 vaccinates, 10 controls). Vaccinations were given at a 21-day interval and half of each group were challenged with BLU1 and half with BLU23 on day 14 after secondary vaccination.

Serology

Fifteen (75%) vaccinates were positive in ELISA by day 11 after primary vaccination and all were positive by day 14 after secondary vaccination (Table 2). No neutralising antibodies were detected in any sheep after either vaccination.

Table 2. Serological status of sheep vaccinated with baculovirus-expressed recombinant bluetongue core-like particles on days after primary and secondary vaccination.

Sheep No.	Serological status					
	Day 11 APV			Day 14 ASV		
	A	E	S	A	E	S
Vaccinates						
1046	+	91	-	++	84	-
1050	+	41	-	+++	117	-
1051	+	84	-	++	91	-
1053	+	90	-	+++	113	-
1056	++	94	-	+++	110	-
1061	+	0	-	++	114	-
1064	++	102	-	+++	90	-
1066	-	0	-	-	111	-
1083	++	65	-	++	81	-
1086	+	0	-	++	103	-
1049	+	69	-	++	98	-
1052	+	0	-	++	83	-
1054	-	0	-	+++	96	-
1055	+	61	-	+++	105	-
1057	+	86	-	+++	104	-
1058	++	66	-	+++	101	-
1060	++	78	-	+++	102	-
1079	++	56	-	+++	136	-
1084	+++	91	-	+++	143	-
1087	++	77	-	+++	96	-
Controls						
1047	-	0	-	-	4	-
1059	-	0	-	-	6	-
1062	-	0	-	-	11	-
1063	-	0	-	-	16	-
1065	-	0	-	-	0	-
1078	-	0	-	-	1	-
1080	-	0	-	-	14	-
1081	-	0	-	-	30	-
1082	-	0	-	-	19	-
1085	-	0	-	-	14	-

A agar gel immunodiffusion test
E ELISA
S serum neutralisation test
APV After Primary Vaccination
ASV After Secondary Vaccination

Temperature

Vaccinated sheep showed no febrile response to challenge with either serotype whereas controls showed a febrile reaction on days 8 and 9 after challenge with both serotypes (Fig. 8).

Haematology

There was no significant difference in TWCC between vaccinates and controls after challenge with BLU1. Control sheep had a significantly lower ($P < 0.01$) TWCC than vaccinates between days 6 and 8 after challenge with serotype 23 (Fig. 9).

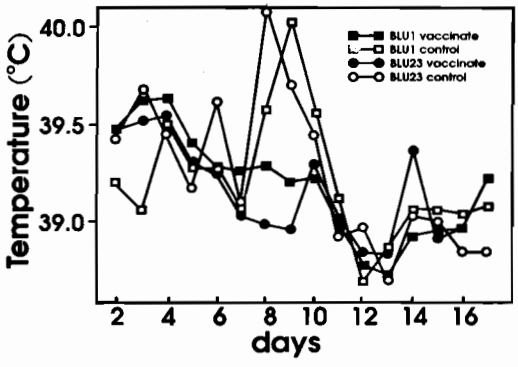

Figure 8. Group mean temperatures after challenge with Australian BLU1 and BLU23 in sheep vaccinated with baculovirus-expressed recombinant BLU core-like particles.

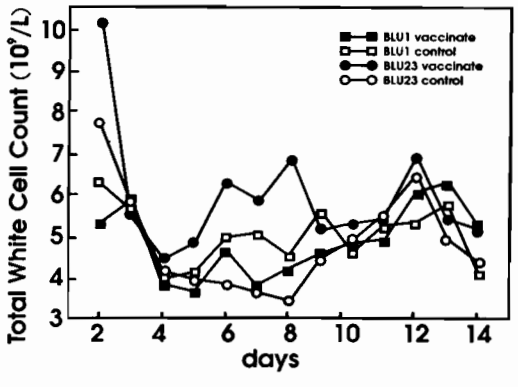

Figure 9. Group mean total leucocyte counts after challenge with Australian BLU1 and BLU23 in sheep vaccinated with baculovirus-expressed recombinant BLU core-like particles.

Postchallenge viraemia

The level of viraemia was significantly lower ($P < 0.01$) in vaccinates than in controls on days 10 and 12 and days 6 to 10 postchallenge with BLU1 and BLU23 respectively (Fig. 10).

Clinical signs

Vaccinated sheep showed no clinical signs of bluetongue following challenge with either serotype. Controls challenged with BLU1 showed mild signs of disease and those challenged with BLU23 showed moderate to severe signs. One (20%) of the controls challenged with BLU23 died of severe disease on day 16 after challenge.

Discussion

Baculovirus-expressed VLPs show VP2 and VP5 in the correct structural configuration, together with VP3 and VP7 in an icosahedral structure identical to that of the BLU virion (French et al. 1990). The production of protective neutralising antibodies in sheep vaccinated with VLPs, in this study and previously (Roy et al. 1992), is strong evidence for the correct presentation of the VP2 antigen to the animal.

Even though vaccination with VLPs afforded good homologous protection, it was unable to inhibit postchallenge viraemia completely. Roy et al. (1992) achieved absence of postchallenge viraemia in sheep vaccinated with 10 µg of VLPs but not in all sheep vaccinated with 100 and 200 µg doses. This inability to limit postchallenge viraemia is in contrast to vaccination with live attenuated BLU (Roy et al. 1992; S.J. Johnson pers. comm.).

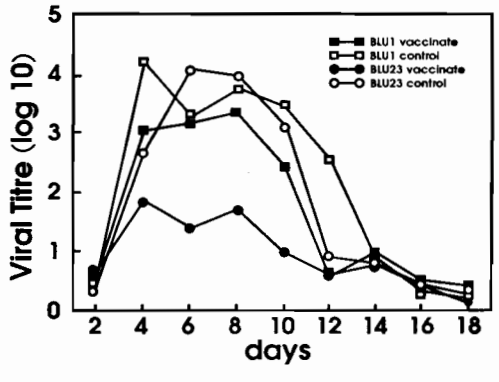

Figure 10. The level of viraemia in sheep after challenge with BLU1 and BLU23.

The ability of vaccination with CLPs to protect sheep in the absence of neutralising antibodies suggests a strong cell-mediated immune response and/or non-neutralising antibodies in protection against bluetongue viruses. In this study any cell-mediated immune response was likely to have been optimal when sheep were challenged 14 days after booster vaccination (Jeggo and Wardley 1982).

These results suggest that vaccination with a combination of VLPs and CLPs may give good homotypic and adequate heterotypic protection. However, the longevity of the heterotypic protection afforded by vaccination with CLPs needs to be ascertained.

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Studies on the Vaccination of Sheep with Bluetongue Virus Core-like Particles Produced by a Recombinant Baculovirus

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Abstract

Bluetongue virus (BLU) core-like particles (CLPs), containing the major structural proteins VP7 and VP3 and expressed by a recombinant baculovirus, were used in a dose-response vaccination study. Six groups of four sheep were inoculated with purified CLPs in ISA-50 adjuvant, and revaccinated after three weeks. Sheep in each group were given either 1, 10, 20, 50, 100 or 200μg CLP protein at each vaccination, while a control group of nine sheep was inoculated with adjuvant in phosphate buffered saline (PBS) alone. Sheep were bled at regular intervals to measure antibody to VP7. Eleven weeks after the first inoculation, all sheep were challenged with a virulent isolate of BLU23. Blood samples were taken to determine levels of viraemia. By 10 days post-challenge, four control sheep had developed coronitis in all feet, and by 14 days all control sheep had body temperatures greater than 40°C, although no mortalities occurred. In vaccinated sheep, fever was the only clinical sign observed. The control group had a mean clinical reaction index (CRI) of 4.1. All vaccinated groups had a reduced mean CRI ($p<0.05$), the greatest being for the 100 μg (CRI 0.82) and 200 μg groups (CRI 2.3). Seventeen sheep from control, 10, 100 and 200μg groups were monitored for viraemia, which was detected in all animals. Compared with the seven control animals, peak viraemia was reduced by 1 $\log_{10}$ or more in three sheep from the 200μg group and by 1 $\log_{10}$ in one sheep from the 10 μg group.

BLUETONGUE virus (BLU) is the causative agent of an insect-transmitted infectious disease of sheep, cattle, goats and wild ruminants. Twenty-four BLU serotypes have been identified to date.

Vaccination is the most cost-effective method of controlling most viral diseases. In the case of BLU infection, only live-attenuated vaccines have proven to be effective. However, the disadvantages of BLU live-attenuated vaccines include risks associated with insufficient attenuation, reversion to virulence, gene reassortment (Gibbs and Greiner 1988) and reproductive losses when vaccinating pregnant sheep (Osburn 1994b). Both attenuated and killed BLU vaccines confer serotype-specific immunity to challenge, with only some cross protection between some serotypes (Stevens et al. 1985; Stott and Osburn 1990). In regions where more than one BLU serotype is endemic, the need to provide immunity against several serotypes presents practical problems. Foremost is the issue of reduced immune responses in sheep vaccinated with multicomponent or multivalent vaccines (Erasmus 1975; Huismans 1985; Raadsma et al. 1994).

Subunit vaccines, carrying the antigenic determinants which afford protective immunity, can be obtained by recombinant DNA technology. These appear to be the most promising means of combining the safety of inactivated vaccines with the efficacy of a monovalent live-attenuated BLU vaccine. One possible vaccine is derived from a recombinant baculovirus containing the cDNA for the two major BLU core proteins, VP3 and VP7 (French and Roy 1990). The expressed product forms BLU core-like particles (CLPs) which are not serotype-specific. This paper reports that partial protection to BLU challenge can be conferred on sheep vaccinated with CLPs.

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Materials and Methods

Origin and purification of CLPs

Spodoptera frugiperda cells in suspension culture were infected by a recombinant *Autographica californica* nuclear polyhedrosis virus containing the dual-transfer vector pAcVC3.BLU-10.7.BLU-17.3 (French and Roy 1990). Forty-eight hours after infection, the cells were harvested, washed in phosphate buffered saline (PBS) and lysed in 0.05 M Tris HCl (pH 8.0) 0.15 M NaCl 0.5% Nonidet P-40. The expressed CLPs were then semipurified by ultracentrifugation through a discontinuous sucrose gradient (30–50% wt/vol in 0.2 M Tris HCl, pH 8.0). Semi-purified BLU CLPs were then purified by ultracentrifugation on a self forming continuous CsCl gradient (44% wt/vol in 0.05 M Tris HCl, pH 8.0) followed by a second CsCl discontinuous gradient (30–44% wt/vol in 0.2 M Tris HCl, pH 8.0). CLPs were checked by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), filtered and concentrated using an Ultrafree-CL filter unit (10 000 NMWL, Millipore). The CLPs were resuspended in PBS and their protein concentration determined by the bicinchoninic acid (BCA) protein assay (Pierce Co.)

Preparation of CLP vaccine

Vaccine was formulated with 10, 100 and 200 μg of CLPs and consisted of a 1:1 emulsion in Montanide Incomplete Seppic Adjuvant (ISA-50; mannide oleate in mineral oil), a gift from Tall-Bennett Pty Ltd, Melbourne, Australia.

Animals

The experimental animals comprised 25 Merino sheep of both sexes, from 2 to 4 years in age. These animals were free of BLU-serogroup antibodies as determined by a competitive enzyme linked immunosorbent assay (cELISA). Nine sheep were used as controls, eight in the 10 μg group and four in each of the 100 and 200 μg groups.

Vaccination procedure

Vaccinated sheep were inoculated with CLPs and adjuvant (1:1) while control sheep received only PBS and adjuvant (1:1). A total volume of 2 mL per sheep was inoculated subcutaneously behind the left or right ear. Three weeks after the initial vaccination the same procedure was repeated.

Vaccine challenge

Eleven weeks after the first vaccination, all sheep were challenged with a single inoculation, subcutaneously at the shoulder, with 1 mL heparinised sheep-passaged blood containing BLU23. This blood, from a sheep that had died from bluetongue, had been titrated in sheep to determine the minimal dose required to induce clinical signs in all sheep (data not presented).

Blood sampling

Clotted blood was collected from all sheep three times a week, from the first day of vaccination until 10 days after the booster. Sheep were then bled at one week intervals until the challenge with BLU23. Heparinised and clotted blood samples were collected on days 5, 7, 10, 12, 14, 21 and 28 post-challenge.

Monitoring of clinical signs

Sheep were monitored daily for body temperature and clinical signs for 14 days following the challenge with BLU23. Clinical signs were quantified by a clinical reaction index (CRI), as described by Huismans et al. (1987). A maximum score of 12 was given to a cumulative total of body temperature of more than 40.0°C from 3 to 14 days post-infection, a score from 0 to 4 each to mouth lesions, nose lesions and foot lesions (lameness, coronitis) and a score of 4 for death. These scores were added, to a maximum possible score of 28.

Serology

Antibody to VP7 was detected by cELISA (Gard and Kirkland 1993). Briefly, 96-well flat-bottom microtitre plates (Greiner medium binding) were coated with 50 mL/well of cell culture derived antigen diluted in 0.1 M carbonate buffer (pH 9.6) and held at room temperature overnight. After washing the plates, 50 mL of a 1:10 dilution of test serum in skim milk buffer (1% skim milk in 0.5% Tween 20 in PBS) was added to duplicate wells. High and low positive and negative standards were included on each plate. The plates were then shaken and incubated at 37°C for 30 min. Following incubation, 50 mL of an anti-VP7 monoclonal antibody (MAb; CSIRO/BLU/20E9B7G2) was added to each well. After incubating the plates again for 30 to 45 min, 50 mL of peroxidase-conjugated goat anti-mouse IgG was added to each well. After incubation and washing 3% TMB substrate was added. The colour reaction was stopped by adding 1M H_2SO_4, the plates read at 450 nm in a microplate photometer and results expressed as percent inhibition (PI%). Values greater than or equal to 40% inhibition were defined as positive.

Titration of antibodies after vaccination

The serogroup antibody response was quantified in some of the sheep testing positive in the cELISA. These included four sheep from the 10 μg CLP vaccinated group and two sheep each from the 100 and 200 μg groups. Sera were diluted in skim milk buffer from 1:10 dilution in doubling dilutions to 1:25600. These dilutions were then tested using the cELISA with the serum antibody titre defined as the last dilution to give 40% inhibition.

Titration of viraemia post-challenge

Heparinised bloods were collected at 5, 7, 10, 12 and 14 days postchallenge from selected sheep (Table 1) and centrifuged. The red blood cells and/or buffy coat were collected and lysed in sterile distilled water (1:1). Appropriate dilutions (10^{-1} to 10^{-6}) were prepared in PBS. Each dilution was injected into five embryonated chicken eggs (ECE) to determine the end-point for each sample. After 5 days the embryonic liver was harvested into a solution containing 1 mL of PBS-Tween (0.5% Tween 20 in PBS) and homogenised. Supernatant (100 mL) from each liver homogenate was passaged into each of two tube cultures of *Aedes albopticus* (AA) mosquito cells when the cells were 50–70% confluent. After one week, 100 mL of a suspension of cells was passaged to duplicate BHK21 cell cultures at 50% confluency. All tubes were examined daily for seven days for cytopathic effects (CPE). Those tubes which showed CPE during that time were stored at 4°C. Tubes which showed no signs of CPE were passaged twice

Table 1. Clinical response index (CRI), viraemia[a] and serogroup antibody (SAb) titres of sheep vaccinated with CLPs and challenged with BLU23.

Group	Sheep no.	CRI	Group Mean CRI	Viraemia 5 DPI	Group mean viraemia 5 DPI	Viraemia 7 DPI	Group mean viraemia 7 DPI	Viraemia 14 DPI	SAb Titre 3 WPIV	SAb Titre 10 WPIV
control	C4	2.6		4.89		3.78		nil	–ve	–ve
	C5	1.8		3.54		3.50		1.50	–ve	–ve
	C7	3.9		5.36		5.02		1.81	–ve	–ve
	C9	4.8		5.20		4.07		1.50	–ve	–ve
	C10	2.2		2.50		4.89		2.09	–ve	–ve
	8A	4.4		nd		nd		nd	–ve	–ve
	8B	5.5		nd		nd		nd	–ve	–ve
	8C	6.4		4.90		4.39		nil	–ve	–ve
	8D	5.9		5.30		4.50		nil	–ve	–ve
			4.1 ± 1.7		4.5 ± 1.1		4.3 ± 0.56			
10 µg	11	1.9		3.60		4.40		nil	1:400	>1:25600
	12	1.8		nd		nd		nd	nd	nd
	13	0.5		nd		nd		nd	nd	nd
	14	4.2		4.13		3.13		nil	1:200	>1:25600
	71	1.3		nd		nd		nd	nd	nd
	72	2.0		1.78		3.54		nil	1:1600	1:25600
	73	2.9		4.42		3.12		2.12	1:1600	1:12800
	74	6.4		nd		nd		nd	nd	nd
			2.6 ± 1.9		3.48 ± 1.2		3.54 ± 0.6			
100 µg	41	0.8		5.50		4.13		nil	1:200	1:12800
	42	1.0		nd		nd		nd	nd	nd
	43	0.4		nd		nd		nd	nd	nd
	44	1.1		4.20		5.89		1.78	1:100	1:12800
			0.82 ± 0.3		4.85 ± 0.9		5.01 ± 1.24			
200 µg	81	1.2		3.66		3.57		2.21	nd	nd
	82	3.2		4.44		4.40		nil	1:400	1:6400
	83	2.6		3.62		3.60		2.54	nd	nd
	84	2.3		5.60		4.44		1.94	1:3200	1:12800
			2.4 ± 0.8		4.33 ± 0.93		4.0 ± 0.48			

[a] Viraemia expressed as $\log_{10}$ chick embryo infectious $dose_{50}$/mL

DPI days post infection
WPIV weeks post initial vaccination
nd not done

in BHK21 cells. After three passages, BHK21 cells that were not showing CPE were considered to be BLU-negative. The BLU titre in the blood was then calculated as $\log_{10}$ chick embryo infectious $dose_{50}$ ($\log_{10}$ $CEID_{50}$)/mL.

Statistical analyses

An approximate Student's t test across groups for the CRI was carried out because of the wide variance within groups. For the peak of viraemia at either day 5 or day 7 post-challenge, a Student's t test was used to analyse group results.

Results

Clinical reaction index (CRI)

Sheep vaccinated with CLPs and challenged with virulent BLU23 had a significant reduction ($p<0.05$) in the severity of clinical signs as measured by their group mean CRI (Table 1). While control sheep showed fever and coronitis, only fever was observed in vaccinated sheep.

Control sheep had a group mean CRI of 4.1 ± 1.7. The CRI for this group represented a fever in all animals exceeding 40°C for 2–5 days and coronitis in four out of nine sheep at seven days after challenge. No other signs were observed and no deaths occurred.

Sheep vaccinated with 10 μg CLPs had a group mean CRI of 2.6 ± 1.9. All sheep from this group manifested fever exceeding 40°C for 1–4 days. For the 100 μg group the group mean CRI was 0.82 ± 0.3, due to fever exceeding 40°C for 1–2 days. The 200 μg group had a mean CRI of 2.4 ± 0.8, with fever exceeding 40°C being observed for 3–4 days.

Within the vaccinated groups there was some individual variation in fever and its duration. In the 100 μg group fever did not exceed 41°C, whereas in five out of eight sheep in the 10 μg group, and in three out of four sheep in the 200 μg group, fever exceeded 41°C. For the control group five out of nine sheep had a rectal temperature greater than 41°C.

Viraemia

Seventeen animals were tested for viraemia. Only the 10 μg group had a statistically significant mean titre ($p<0.05$) lower than the titre for the control group (4.3 ± 0.56 $CEID_{50}$) at 7 days post-infection (Table 1). However, at seven days after challenge most vaccinated sheep (6/10) had titres less than the mean for the control animals. Two animals (14 and 73) from the 10 μg group were approximately two standard deviations less than the mean for the control group, and a further three (no. 72 from the 10 μg group, and 81 and 83 from the 200 μg group) were at least one standard deviation less.

Vaccination did not affect when viraemia peaked or its duration. Thirteen animals (7/10 vaccinated and 6/7 controls) had a peak viraemia by day 5, while the remaining four peaked by day 7. Viraemia was undetectable in five animals (4 vaccinated, 1 control) by day 12, and by day 14 in three animals (1 vaccinated, 2 controls). Nine animals (5 vaccinated, 4 controls) were still viraemic at 14 days after challenge.

Serogroup antibody titration

Sera from all vaccinated sheep were tested by cELISA at the standard dilution of serum (1:10) after the first and second vaccinations for eight weeks to determine the serogroup antibody response. All vaccinated sheep became positive by 2 weeks after the initial vaccination.

The antibody titres of eight vaccinated sheep (four sheep from 10 μg group, two sheep from 100 μg group and two sheep from 200 μg group) were determined by cELISA (Table 1). There was a dramatic increase in antibody titres after the booster vaccination.

There was a negative correlation between the dose of CLPs and the antibody titre. One week before virus challenge, the titres for the 10 μg group were 1:25600 for three of the four sheep (one sheep 1:12800), 1:12800 for sheep in the 100 μg group, and 1:12800 and 1:6400 for sheep in the 200 μg group.

Overall, then, in this study sheep in the 10 μg group had the highest antibody titre, the lowest titre of viraemia but the least reduction in CRI. In the 100 μg group, sheep had intermediate antibody titres, the highest titre of viraemia but the greatest reduction in CRI. Sheep in the 200 μg group had the lowest antibody titre, moderate titres of viraemia (closest to the control group) and a moderate reduction in CRI.

Discussion

The vaccination of Merino sheep with recombinant baculovirus expressed CLPs resulted in a significant reduction ($p<0.05$) in clinical signs after challenge. This reduction was primarily due to the absence of coronitis and a reduction in the degree of fever in vaccinated sheep compared to controls.

Although there was a reduction in viraemia up to 2 $\log_{10}$ $CEID_{50}$ for some individual vaccinated animals, this reduction was not consistent on a group basis, and for most groups the mean reduction was not significant. The statistically significant reduction in viraemia observed in the 10 μg group will need further investigation to determine whether this is reproducible, as only small numbers of animals were included in each group. Interestingly, there was no correlation between the titre of viraemia and the clinical response after challenge.

Titres of antibody to VP7 appeared to be inversely proportional to the quantity of CLPs inoculated into the sheep. The highest titres were found for animals within the 10 μg groups: animals inoculated with 100 mg and 200 μg CLPs had lower titres of anti-VP7 antibody as determined by the cELISA. Furthermore, maximum titres did not occur until after the second vaccination.

CLPs have been proposed as having potential as a broad spectrum BLU vaccine. Our results demonstrate some of this potential. Faced with an outbreak of BLU infection, where type-specific vaccines were not available or the serotypes unknown, CLPs could be used to ameliorate the impact of the infection. However, the reduction in viraemia observed in vaccinated animals may not be sufficient to help control spread of the infection. Nevertheless, any amelioration in disease would have both economic and animal welfare benefits, in terms of reduced mortalities and increased growth rates and wool production.

In contrast to live attenuated vaccines, CLP vaccines have several advantages conferred by their non-infectious nature. CLP vaccines do not have the risks inherent in all live vaccines of reversion to virulence, insufficient attenuation, genetic reassortment and a range of reproductive losses and disorders (Gibbs and Greiner 1988; Van Dijk 1993; Osburn 1994b). Reversion to virulence by vaccine viruses, or genetic reassortments, are a real risk in endemic areas. Vaccination during an outbreak in these regions is not recommended because of the possibility of passaging attenuated viruses through insects (Doel 1993), or of reassortment between attenuated serotypes and/or field isolates. For these reasons, existing vaccines should be administered out before the beginning of seasonal vector activity (Osburn 1994a), whereas subunit vaccines, such as CLP vaccines, can be used at any time. CLPs could also be engineered to distinguish between vaccinated and BLU-infected sheep by the development of an appropriate ELISA, following a similar approach to that developed for rinderpest (Ismail et al. 1993). Multivalent BLU vaccines may also produce immunological interference or antigenic competition (Huismans 1985). Vaccination by CLPs may be a temporary yet effective approach until this problem is overcome.

It is well recognised that anti-VP7 antibody does not protect against BLU infection. The apparent reduction in clinical disease following vaccination with CLPs suggests that other immune mechanisms are important in ameliorating the clinical impact of BLU infection in vaccinated animals. Studies in progress are investigating the role of cell-mediated immunity in affecting the clinical outcomes induced by CLP vaccination. CLPs themselves could be useful probes for sheep immune responses to BLU infection.

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Estimations of the Divergence of Bluetongue Viral Populations in Indonesia on the Basis of Virus Isolation and PCR Sequence Analysis

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Abstract

In Indonesia, seven serotypes of bluetongue virus (BLU) have been isolated, mostly from the blood of sentinel cattle but also from *Culicoides* vectors and mosquitoes. Three serotypes have also been isolated from locations some 4000 km apart in eastern and western Indonesia. How mobile are virus populations in such a large country? Does each virus serotype comprise one large homogenous national population or are there detectable differences at the molecular level among isolates from different sources? Are there detectable differences from a single source over relatively short periods of time? Differences may indicate separate subpopulations of viruses, and give a means of studying aspects of their epidemiology. This study compared the nucleotide sequences of specific regions of the viral genomes of Indonesian isolates of bluetongue virus amplified by polymerase chain reaction (PCR).

BLUETONGUE viruses (BLU) are members of the *Orbivirus* genus of the family Reoviridae. Twenty-four BLU serotypes have been defined by their reactions in serum neutralisation tests (Gorman et al. 1983). The viruses contain ten double-stranded segments of RNA within a double-layer capsid. The inner capsid comprises two major proteins, VP3 and VP7, and three minor proteins, VP1, VP4 and VP6. The outer capsid comprises two proteins, VP2 and VP5 (Verwoerd et al. 1970, 1972; Huismans 1979). Non-structural proteins, NS1, NS2 and NS3, have also been identified (Mertens et al. 1984; Eaton and Gould 1987).

Serological studies have shown that bluetongue viruses are widespread throughout the islands of Indonesia (Sendow et al. 1986, 1991a). Reactors were found in cattle, buffaloes, goats and sheep sera collected from all the major islands. In West Java, monitoring of sentinel cattle in 1987 and 1988 yielded arboviral isolates which were subsequently confirmed as BLU7 and 9 (Sendow et al. 1991b). At this site in 1990, five BLU serotypes were isolated from sentinel cattle over a six month period (Sendow et al. 1993a, c). In Irian Jaya, 4000 km to the east of the West Java site, similar serotypes were isolated during the same period, suggesting that these viruses were distributed in widely separate areas of Indonesia (Sendow et al. 1993b). How mobile are these viral populations in such a large country? Does each serotype comprise one large homogenous national population or are the isolates representatives of smaller populations from different sources?

To address such questions for orbiviruses, including BLU, techniques have been developed to analyse molecular differences among isolates. Nucleotide sequences of relatively conserved regions of viral RNA gene segments can be analysed (Gould et al 1989; Gould and Pritchard 1991). By focussing on RNA gene segment 3, coding for the inner capsid protein VP3, it has been shown that BLU viruses fall into distinct topotypes, and that within topotypes there is further variation correlating with geographic region of isolation.

The aim of this study was to compare the sequence analyses of RNA segment 3 (Gould and Pritchard 1991) of bluetongue isolates with their geographic origin within Indonesia for epidemiological studies. On the basis of such knowledge it may be possible to investigate further the relationships among Indonesian viruses and those from other countries in the region, especially northern Australia.

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Australia and Indonesia have had co-operative programs in veterinary arbovirology for several years, incorporating both virus and vector studies (Daniels et al. 1995). This work enhances Australian preparedness for the possible entry of new viral serotypes and insect vectors into Australia, while Indonesia benefits from a greater understanding of its animal health status. The information also helps stabilise trade in livestock and livestock products, contributing to the prosperity of both countries.

Geographic Distribution of Serotypes

Serological studies

Sendow et al. (1991a) reported on the first national serological survey for several BLU serotypes, using sera stored in a serum bank (Young et al. 1985). Reactors were detected to BLU1, 12, 17, 20 and 21, although overall prevalences to BLU1, 12 and 17 were low (< 5%). Since titres of these sera were also low (< 1:16), and most also reacted with other serotypes at higher titre, cross-reactions were considered a possibility for these three serotypes. However, previous localised studies in two provinces, East Java and Bali, had detected high prevalences of antibodies to BLU1 and 20, and a low (< 5%) prevalence of reactors to BLU12 (Miura et al. 1982). Overall the serological data from the two studies indicated a high probability of infections with BLU1, 20 and 21, with the majority of the 24 BLU serotypes untested.

Sendow et al. (1991a) detected reactors to BLU1 only in Java, with BLU 20 and 21 reactors being detected on all islands studied, from Sumatra in the north-west through Java, Kalimantan, Sulawesi to Timor in the south-east.

Viral isolations

The original isolations of BLU7 and 9 were from Depok in West Java. Subsequently, BLU1, 12, 21 and 23 were isolated from sentinel cattle at the same site, as well as a second isolate of BLU9 (Sendow et al. 1993a). These results substantiated the observation of antibodies to BLU1 and 12 , although there have been no further serotype-specific serological studies. Hence bluetongue virus serotypes confirmed in Indonesia are BLU1, 7, 9, 12, 21 and 23, with serological evidence for BLU20. Recently, further BLU isolates from West Java have been identified, and an isolate of BLU3 has been confirmed (Sendow unpublished data).

Although sentinel cattle have been monitored for virus isolation from other sites, including Bali, West Timor and two sites in Irian Jaya (Daniels et al. 1995), bluetongue viruses have been confirmed from only one other district, Jayapura in Irian Jaya. There BLU1, 21 and 23 were reported (Sendow 1993b) with an isolate of BLU16 recently confirmed (Sendow unpublished data). This finding of similar serotypes in the east as well as the west of Indonesia (Fig. 1) is of great importance epidemiologically. The finding of BLU1 in both locations indicates a broad distribution of this serotype, contrary to the previous serological evidence. The results also support the widespread distribution of BLU21, as suggested by the serological data, and indicate that an additional serotype, BLU23, may have a similarly wide distribution. Apart from these shared serotypes, the results have identified serotypes presently unique to each location, BLU3, 7, 9 and 12 in West Java and BLU16 in Irian Jaya. Despite serological evidence that BLU20 is widely distributed, so far there has been no confirmed isolation of this serotype in Indonesia.

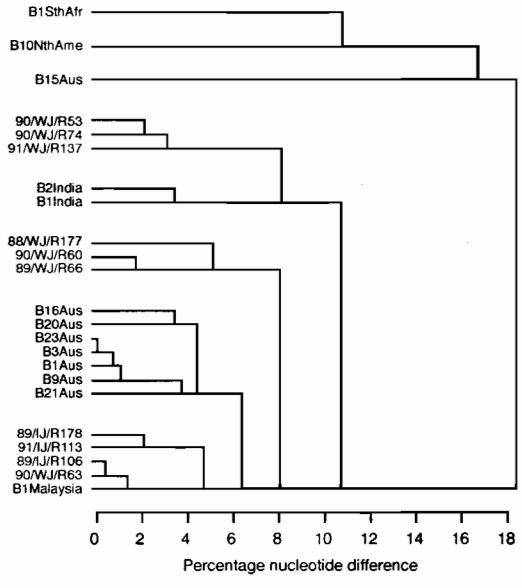

Figure 1. Dendrogram of genotypic relationships among Indonesian bluetongue virus (BLU) isolates.

Hence bluetongue viruses in Indonesia can be considered as three groups based on site of isolation (Table 1): those found only in the west, those found only in the east, and those found in both. A primary objective of the molecular studies was therefore to investigate whether these groupings were supported by sequence analysis of the RNA 3 gene segment. Also, since western and eastern Indonesia are adjacent to different land masses, the question arose as to whether the Indonesian viruses comprise one or more isolated populations, or whether they are shared in broader pools among different countries in close geographical proximity.

Table 1. Distribution of bluetongue virus (BLU) serotypes in Indonesia.

BLU serotype isolated	Provinces affected	
	West Java	Irian Jaya
BLU1	+	+
BLU3	+	
BLU7	+	
BLU9	+	
BLU12	+	
BLU16		+
BLU21	+	+
BLU23	+	+

Materials and Methods

Viruses and nucleic acids

Bluetongue virus isolates (Table 2) were grown in baby hamster kidney (BHK21) cells and nucleic acid was extracted as described by Eaton and Gould (1987). Complementary DNA (cDNA) copies of dsRNA were generated using AMV reverse transcriptase (Promega) as described previously (Pritchard et al. 1995), and BLU VP3 gene-specific primers as described by McColl and Gould (1991).

Polymerase chain reaction and sequencing

Polymerase chain reaction (PCR) amplification from cDNA was done using the GeneAmp DNA reagent kit (Perkin-Elmer Cetus) following manufacturer's recommendations (Saiki et al. 1988) with minor modifications (McColl and Gould 1991). Procedures were carried out in a final volume of 50 mL for 25 cycles of denaturation, 94°C for 1 minute, annealing, 37°C for 2 minutes and elongation at 72°C for 2 minutes. An aliquot of 50 mL of each PCR was analysed in a 1.8% agarose/TAE buffer gel containing ethidium bromide. After electrophoresis, the DNA fragments were visualised under ultraviolet light. Nucleotide sequencing of PCR products was done by the dideoxynucleotide chain termination method with Sequenase (USB, Amersham) using their PCR Sequencing Kit according to the instructions provided.

Computer analysis of nucleotide and amino acid sequences

Nucleotide sequence alignments were done with the ALIGN Plus program Version 2.0 (Scientific & Educational Software): SEQPROG (Knowles, unpublished) was used to compare the sequences using DNADIST and KITSCH programs from the PHYLIP package (Felsenstein 1985) and to give a single most parsimonious, unrooted tree.

Results and Discussion

Table 2 summarises data on the Indonesian BLU isolates included in this study, the collection sites, date of collection, and serotype. After isolation in cell culture, isolates were analysed by PCR and sequencing. Nucleotide sequence homology among these Indonesian BLU isolates using partial VP3 gene sequences was calculated: homologies varied between 88% and 100% identity depending on which isolates were compared (Table 3). Isolates were grouped on the basis of sequence homologies greater than 95%.

All the partial VP3 gene sequences of these Indonesian BLU isolates belonged to the Australasian topotype or regional grouping, as distinct from the South African and North American groupings and the special group comprising the Australian BLU15 (Gould 1987; Pritchard et al. 1995). Within the Australasian topotype, further groupings of these Indonesian isolates were observed, even from the same collection site. Three such groups were identified in which VP3 gene sequences differed by only 5% within each group, while there was an 11% difference between groups (Fig 1).

Table 2. Grouping of bluetongue virus (BLU) isolates in Indonesia, based on nucleotide sequence analysis.

Isolate	BLU serotype	Source	Date	Sequence group
RIVS 53	3	West Java	March 90	A
RIVS 74	9	West Java	January 90	A
RIVS177	9	West Java	March 88	B
RIVS 66	21	West Java	December 89	B
RIVS 60	21	West Java	April 90	B
RIVS 63	21	West Java	June 90	C
RIVS137	21	West Java (insect)	May 91	A
RIVS113	21	Irian Jaya	February 91	C
RIVS106	23	Irian Jaya	November 89	C
RIVS178	16	Irian Jaya	December 89	C

Table 3. Nucleotide sequence homology among the Indonesian bluetongue viral RNA 3 gene segments over the region 1398–1693bp.

	90/WJ/ R63	90/WJ/ R66	90/WJ/ R74	89/IJ/ R106	91/IJ/ R113	91/WJ/ R137	88/WJ/ R177	88/IJ/ R178	90/WJ/ R53	90/WJ/ R60
90/WJ/R63	.....									
89/WJ/R66	92	.....								
90/WJ/R74	90	87	.....							
89/IJ/R106	100	92	90	.....						
91/IJ/R113	96	93	89	96	.....					
91/WJ/R137	90	90	97	90	91	.....				
88/WJ/R177	93	96	89	92	92	91	.....			
88/IJ/R178	96	92	90	96	98	90	93	.....		
90/WJ/R53	89	88	98	89	90	98	89	90	.....	
90/WJ/R60	91	98	88	91	93	90	95	92	89	.....

In group A, isolate RIVS 137 was obtained from a mixed pool of *Culicoides (Avaritia)* species collected in May 1991 in West Java. The other isolates (RIVS 53 and 74) were from cattle blood collected in 1990. In this group, the sequence of the insect isolate did not differ significantly from those of the blood isolates. Group B comprised RIVS 60, 66, and 177 from West Java, isolated from cattle blood between 1988 and 1990. Group C consisted of three isolates from Irian Jaya (RIVS 106, 113, and 178) and one from West Java (RIVS 63). These Indonesian group C isolates were found to group with an isolate from Malaysia studied previously (Pritchard et al. 1995).

The groupings were independent of serotype (Table 2). Isolates of BLU21 were represented in each of groups A, B and C, and the two isolates of BLU9 from West Java occurred in groups A and B. Although the period of time represented by the isolates was too short to allow identification of significant temporal trends, both of the earlier (pre-1990) isolates in West Java (RIVS 177 and RIVS 66) were in group B, while the third group B isolate (RIVS 60) overlapped the group A isolates in 1990. The group C isolates spanned the period November 1989 (RIVS 106) to February 1991 (RIVS 21).

Hence, even within a short time period (1988–1991), a range of VP3 genome sequence-types were observed circulating within the islands of the Indonesian archipelago. While in West Java there were two circulating groups of viruses with similar VP3 genome sequences identified only at that location, another group (group C) contained BLU isolates from Irian Jaya, Malaysia and West Java (Fig. 1).

Since the molecular analyses indicated that the viruses in Irian Jaya were homologous with isolates from West Java and Malaysia, the possibility is raised of a common pool of isolates deriving from mainland Southeast Asia and spread throughout the island chain. However this suggested picture was confounded by the simultaneous identification of a further two distinct groupings of bluetongue viruses in West Java. For these groups of isolates to be genetically distinct, we may hypothesise prolonged periods of separation of these groups, allowing divergent evolution. This would then imply that at least some of the three groups identified in West Java have been introduced from elsewhere, or that different groups of viruses can evolve separately in the same geographic area.

Routes of suspected vector and arboviral movements have been frequently hypothesised (Dyce 1982; St. George 1986; McColl et al. 1994). Molecular epidemiological studies of virus isolates have the capability to identify differences among isolates of the same virus from different geographic regions (Trent et al. 1983). Differences have been shown between Australasian, South African and North American BLU isolates based on gene sequences of the conserved core protein VP3 (Gould and Pritchard 1990; Pritchard et al. 1995), and hence inferences made on the geographic origin of isolates showing similar sequences (Gould 1987; Gould and Pritchard 1990; Pritchard et al. 1995).

None of these Indonesian isolates grouped with the prototype viruses from northern Australia, isolated during the period 1975 to 1986 (Gard and Melville 1989). Hence the Indonesian viruses seem to represent populations separate from the early Australian isolates, although since the rate of evolutionary change in sequence is unknown for these viruses, the effect of time on the variability of the genotypes of these viral populations cannot be assessed with confidence.

Much remains to be learned about the relationships among bluetongue viruses in the Australian and Southeast Asian regions, especially in Indonesia where traffic in livestock, and so presumably in insect vectors, continues between the western and eastern regions.

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Molecular Epidemiology of Bluetongue Serotype 10 Virus from the Western United States

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Abstract

Bluetongue viruses (BLU) have segmented genomes which are capable of reassorting with other strains and serotypes. Previous studies have utilised monoclonal antibodies, oligonucleotide fingerprinting, electropherotyping and nucleic acid hybridisation to demonstrate genetic heterogeneity. In this study, BLU serotype 10 field isolates from 1980–81 and 1990 were compared to prototype BLU2, 10, 11, 13 and 17 and BLU10 vaccine virus by sequence analysis. The results indicated that there were two major phylogenetic groups of both gene segments 2 and 9. Furthermore, the more conserved gene segment 9 was found to reassort with all serotypes. Prototype BLU13 virus was found to have gene segment 9 that was more similar to gene segment 9 from the BLU10 modified live virus vaccine which was in use 12 years before the prototype BLU13 virus was isolated. This suggests prototype BLU13 from the United States is a reassortant virus with gene segment 9 derived from BLU10 vaccine virus.

BLUETONGUE is an arthropod-borne viral disease of ruminants. The viruses causing the disease belong to the genus *Orbivirus*. Four different serotypes of bluetongue virus (BLU10, 11, 13 and 17) have been observed in western United States (Stott et al. 1981). The presence of the viruses is limited by the distribution and habitat of the vector *Culicoides variipennis* var. sonorensis in western United States (Tabachnick 1991). As this species is adapted to human habitats, infections are seasonal, being most common in ruminants in late summer and autumn. Sheep, pronghorned antelope, desert bighorn sheep and whitetailed deer are most susceptible to clinical disease (Jessup 1985). Cattle are commonly infected but rarely show clinical signs (Anderson et al. 1985). The clinical expression of disease in sheep, antelope and deer is associated with viral-based vascular lesions that lead to local thromboses and infarcts of tissues in sheep and disseminated intravascular coagulopathy in the free-ranging ruminants. In cattle, the clinical expression of disease is associated with an IgE-mediated hypersensitivity (Anderson et al. 1985). Congenital BLU infection in sheep and cattle may result in hydranencephaly or porencephaly in sheep and cattle and retinal dysplasia in lambs (Osburn et al. 1971; MacLachlan and Osburn 1983).

Evidence of strain differences in virulence and pathogenic characteristics of viruses of BLU serotypes became apparent during surveillance surveys for prevalence of infection in ruminants. BLU was occasionally isolated from sheep flocks that had no evidence of clinical disease (Stott et al. 1985). Electropherotyping of BLU isolates indicated that there was considerable variation in genome migration patterns within and between serotypes (De Mattos et al. 1991). Further, studies with plaque-picked BLU11 isolates with different electropherotypic patterns, designated UC2 and UC8, indicated a difference in virulence and pathogenic properties of these viruses in newborn Balb/c mice and foetal calves (Waldvogel et al. 1987, 1992). The potential for genetic polymorphism in the BLU serogroups seemed apparent.

Genetic polymorphism is associated with differences in antigenic properties, vector susceptibility, tissue tropism and virulence exhibited by individual members of the same virus group or serotype. Even viruses isolated at the same time and from a common geographical region may be genetically heteroge-

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nous, as may RNA viruses isolated from a single outbreak of disease or even from a single infected host (Sobrino et al. 1986). The natural replication cycle of bluetongue viruses, which includes insect vectors and various ruminant and carnivore hosts, offers significant opportunity for the generation or selection of genetically novel viruses (Roy 1988; Samal et al. 1987; Squire et al. 1983). The evolution and genetic variation of BLU in a restricted geographic region was studied by sequencing and phylogenetic analyses of the most variable gene segment (gene segment 2), and of one of the more conserved genome segments (gene segment 9) of field isolates of BLU10 and 17 obtained from the San Joaquin Valley of California in 1980, 1981 and 1990.

Materials and Methods

Viruses

This study involved BLU10 isolated from ruminants in different locations in California between 1980–1981 and 1990; prototype viral strains of the four serotypes in western USA; and a BLU10 virus vaccine strain (Table 1). The viruses were initially isolated in embryonated chicken eggs and propagated in BHK21 cells (Heidner et al. 1991). Viral dsRNA was extracted with phenol, precipitated with ethanol, and purified with lithium chloride (De Mattos et al. 1989).

PCR amplification and cycling sequencing

The positive and negative strands of gene segments 2 and 9 of the viruses were reverse transcribed and amplified as previously described by Akita et al. (1992) using different combinations of primers; BLU10 L-2 and BLU10 S-3. Primers were constructed from the published sequence of gene segments 2 and 9 of prototype strains of BLU10, 11, 13 and 17 (Ghiashi et al. 1987; Purdy et al. 1985; Fukusho et al. 1989). The cDNA was purified and sequenced by cycle sequencing using a commercial kit (f-mol DNA Sequencing System, Promega) as described by De Mattos et al. (1994a). Each sequencing reaction was repeated several times with PCR products from several different amplifications to control artefacts, and using different combinations of primers to obtain overlapping PCR products that together represented the entire nucleotide sequence of gene segments 2 and 9 of each virus. The Wisconsin Package was used to analyse the nucleotide sequences and to predict the amino acid composition of the putative proteins (Anon. 1991). Phylogenetic analysis was accomplished using the DNADIST and FITCH programs of the PHYLIP package Version 3.4 (Felsenstein 1991). Gene segment 2 of EHD-1 was used as an outgroup as previously described (De Mattos et al. 1994b). The nucleotide sequence corresponding to the 5' and 3' primers used to amplify the genes were not included in the phylogenetic studies. Bootstrap analysis of 100 replicates was performed as described by De Mattos et al. (1994b). A value greater than 95% was considered significant.

Results

Gene segment 2 of all BLU10 isolates comprised 2926 base pairs indicating no insertions or deletions over a 37-year period. The genes coded for 956 amino acids. Gene segment 2 of the prototype strain, the

Table 1. BLU isolates from the United States of America.

Serotype	Field isolate or prototype	Species of origin	Year of isolation	County of isolation	State
BLU10	10O80V	Ovine	1980	Tehama	California
	10B80Y	Bovine	1980	Tulare	California
	10O80Z	Ovine	1980	Solano	California
	10O90H	Ovine	1990	Mendocino	California
	10B90Z	Bovine	1990	Imperial	California
	10O90Z	Ovine	1990	Chino	California
	Vaccine 10	Ovine	1953		California
	Prototype 10	Ovine	1953		California
BLU13	Prototype 13		1967		Idaho
BLU11	Prototype 11		1955		Texas
BLU17	Prototype 17	Bovine	1962		Wyoming
BLU2	Prototype 2		1983		Florida

modified live virus vaccine strain derived from the prototype virus and the 1980 field isolates differed only by 0.1–0.5%. In contrast, the 1990 field isolates constituted a separate group with similarities of 98.2–99.7% between each other, but a 4.8% divergence from the previously described BLU10 viruses. The vaccine strain had nine mutations when compared to the prototype virus, five of which (bases 497, 954, 998, 1012 and 1415) were unique to VAC 10. Four of the mutations (bases 76, 836, 1056 and 1754) were shared with all the field isolates. There were only five exclusive mutations (bases 690, 1836, 2161, 2212 and 2851) among the three 1980 isolates and the prototype virus isolated 37 years earlier. In contrast, the 1990 isolates had 102 nucleotide changes as compared to the prototype virus. These results indicate two distinct consensus sequences of BLU10 viruses in California over the last 37 years. Despite differences in nucleotide and amino acid sequences among these viruses from 1953 to 1990, the general characteristics of the hydropathic profiles and secondary structure of their VP2 proteins remained similar.

Phylogenetic analyses of gene segment 2 of BLU prototypes and BLU10 field isolates (Fig. 1) indicated two distinct monophyletic groups, one including BLU2 and BLU13 and the other including BLU10, 11 and 17 and the field isolates from California. The monophyletic grouping of BLU10, 11 and 17 was supported by common deletions present in the nucleotide sequences of gene segments 2. Additional deletions are present between prototype strains of BLU10 and 11, and between positions 1827–1828 and 1916–1917 of prototype BLU17. The common deletions of the gene segment 2 of BLU10, 11 and 17 suggest that these deletions occurred in a common ancestor before the divergence of each serotype.

The Californian BLU10 viruses under study are derived from a common ancestor from which two different lineages diverged resulting in two monophyletic groups. One group consisted of the prototype, vaccine and 1980 field isolates, and the other the 1990 field isolates. The prototype, VAC 10 and 1980 viruses formed two sister groups. The 1990 group of three included strain 10O90H which appeared to diverge earlier and followed a different evolutionary pathway. Isolates 10O90Z and 10B90Z shared the same immediate ancestor. The varied differences in sequences suggest that the viruses were derived from different evolutionary pathways within the lineage.

Phylogenetic analyses of gene segment 9 of BLU10 isolates and prototype viruses (Fig. 2) indicated that BLU10 field isolates belong to two lineages, one consisting of BLU13, VAC 10, BLU10 and the 1980 field isolates, and the other lineage consisting of the 1990 field isolates, BLU11 and BLU17 prototype viruses. The bootstrap values of each node were 79% and 80% respectively, which does not support the topology of the phylogenetic tree. This indicates that genome segment 9 from the viruses under study could not be separated in serotypes with a 95% level of confidence.

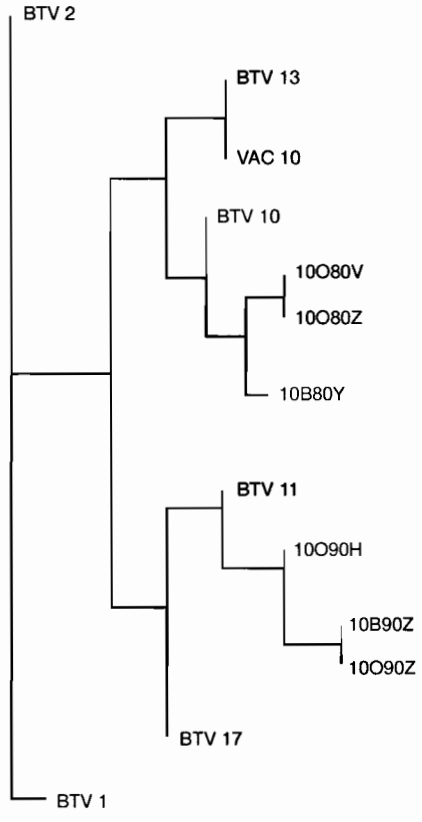

Figure 1. Phylogenetic tree of genome segment 2 of six BLU10 isolates, five USA BLU prototype strains and MLV VAC 10 (EHD1 used as an outgroup).

The prototype BLU13 genome segment 9 was highly homologous to the BLU10 (99.51%), VAC 10 (99.62%) and two of the 1980 field isolates (99.43% and 99.29%). The S3 gene of the 1990 field isolates was more closely related to BLU11 (between 98.5 and 98.4%) than to BLU10 (between 97.3 and 97.4%). Field isolates 10O90Z and 10B90Z were identical, but the field isolate 10O90H was only 97.93% related to the others. The deduced VP6 of BLU13 differs in only two amino acids (position 41 and 199) from the VP6 of VAC 10. These two S3 genes share a methionine codon at position 5, not present in the others, and they lack the third in-frame ATG initiation codon present in all other serotype 10 strains.

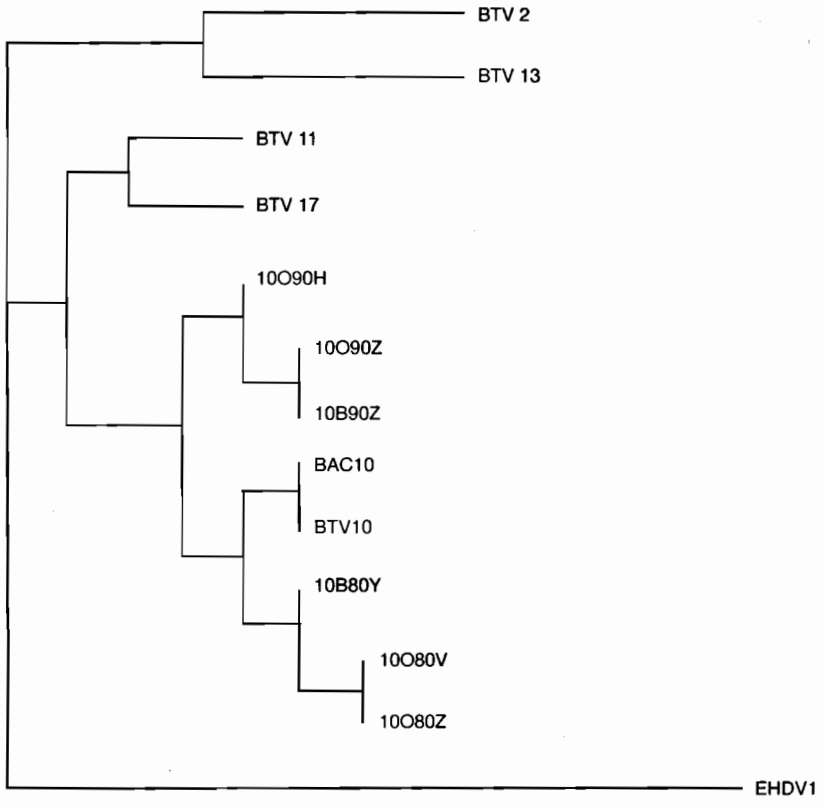

Figure 2. Phylogenetic tree of genome segment 9 genes of six BLU10 isolates, five USA BLU prototype strains and MLV VAC 10.

Discussion

Genetic variability is a common characteristic of many RNA viruses. This genomic heterogeneity may form complex quasispecies populations that influence mammalian and vector preferences, viral ecology, virulence, pathogenesis and antigenic drift. In this paper we have attempted to begin identifying genomic variability and to associate it with phenotypic expression of the viruses, by comparing the variable gene segment 2 and more conserved gene segment 3 of U.S. prototype BLU viruses, and BLU10 field isolates.

The genomic analyses of the gene segment 2 of the viruses examined showed there were two major monophyletic groups of BLU in the USA, comprising BLU2 and 13, and BLU10, 11 and 17. In the case of BLU10, two distinct groups of viruses were observed. The first group is formed by BLU10 prototype strains, the 1980 field isolates and the vaccine strain (VAC 10, derived from the first isolate of BLU obtained in 1953; Jessup 1985). The 1980 field isolates presented only 0.1–0.5% of divergence from the prototype and vaccine viruses. The second group is constituted by the 1990 field isolates. These viruses differed from the BLU10 prototype 10, VAC 10 and 1980 isolates by 4.8%. Although BLU10 isolates from California had a common ancestry, the 1953 and 1980 viruses were sufficiently distinct from the 1990 isolates to indicate that there had been at least two monophyletic BLU10 lineages present in California over the last 37 years. Furthermore, there was no proof that the VAC 10 vaccine virus had influenced the evolution of gene segment 2 of the 1990 viruses. No BLU10 isolates had been made before 1990 in southern California, unlike some of the 1990 isolates. These viruses were of a different lineage from those observed in California before 1990.

Gene segment 9 from the 1980 BLU10 field isolates formed a group that differed in their nucleotide sequences by only 0.08–0.38% from the BLU10 prototype and VAC 10. This contrasted with the 1990

field isolates which were more closely related to BLU11 (98.5% and 98.4%) than to BLU10 (97.3% and 97.4%). The data suggest that genome segment 9 of BLU10 isolates from California is derived from two different ancestors, probably evolving in different geographical areas. Also of interest was the finding that gene segment 9 of BLU13 prototype was highly homologous to VAC 10 (BLU10), suggesting that reassortant genes were present in these virus isolates. The appearance of the VAC 10 gene segment 9 with a methionine marker in the BLU13 prototype virus strongly suggests that the genes from VAC 10 virus and a BLU13 field isolate virus reassorted in a biological system before the initial isolation of BLU13 prototype virus in 1962. A precursor to the VAC 10 vaccine (Blucine, an embryonated egg adapted vaccine) was widely used from 1955 to 1970 in western United States. It is very likely that a BLU13 virus reassorted with the 'Blucine' vaccine virus, and the Blucine gene segment 9 was incorporated into the BLU13 virus, now considered the prototype virus.

Although BLU viruses are closely related, the segment 2 gene of each bluetongue virus was found to be distinct and their mutations have not followed a linear pattern of accumulation over time. This indicates that genome segment 2 of individual viruses might evolve via different evolutionary pathways. These studies further demonstrate that the gene segment 2 population of BLU field isolates are formed by nonidentical but closely related genomes as occurs with the genomes of other RNA viruses.

The phylogenetic analyses of the S3 gene of BLU10 field isolates demonstrated that, despite the conserved nature of this gene, genetic variants are present in the natural BLU population. The clear separation in serotype was not supported by the statistical analyses of their phylogeny. This suggests that the S3 population may be formed by a spectrum of viral variants closely related to a consensus sequence that evolved to its maximum fitness for the virus replication in nature. The stability of this consensus sequence in nature is facilitated by reassortment events that assure the efficient distribution of the most fitted variants among serotypes.

In arbovirus ecology, the vector, host and environment are in a dynamic equilibrium with the temporary or long-term predominance of the viral consensus sequence best suited to the prevalent conditions. Any change in the biological environment that disrupts this equilibrium allows different viral variants to become dominant.

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Control of Bluetongue Disease with Attenuated Vaccine

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Abstract

In 1987, an outbreak of bluetongue occurred in sheep on a farm in Wuxi country, Sichuan Province. Of the 472 sheep, 143 (30.3%) exhibited disease and 93 (65%) of these died. Bluetongue antibodies were found in animals and bluetongue virus (BLU) was isolated at the Yunnan Tropical and Subtropical Animal Virus Disease Laboratory from blood and tissue samples. From 1989 to 1994, a combined attenuated virus vaccine (serotypes BLU1 and 16) prepared at that laboratory was used to control bluetongue disease. Over six years 36,523 sheep were vaccinated. Of those vaccinated only with BLU16 vaccine, 98/3184 (3%) were susceptible to naturally acquired bluetongue in contrast to 67/148 (45.7%) of unvaccinated controls. Seven sheep developed bluetongue in 1989, but none did so in 1990, 1992 and 1994.

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Using Inactivated Vaccine to Prevent Bluetongue in Hubei Province, China

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Abstract

In 1989 an outbreak of bluetongue on a sheep farm in Xiangfan, Hubei Province, caused serious economic loss: 1505 (81%) of the 1837 sheep showed disease, and 521 (34%) of these died. From 1990 to 1994, agar gel immunodiffusion (AGID) was used to distinguish seropositive sheep from seronegatives. The seropositive sheep were eliminated. As a preventative measure, the seronegative sheep were immunised with an inactivated vaccine made at the Yunnan Tropical and Subtropical Animal Virus Diseases Laboratory. This vaccine successfully reduced losses.

USING inactivated vaccine to prevent bluetongue in Hubei, one of China's epidemic bluetongue areas, has been justified as an effective strategy by economic data from 1990–1994 and epidemiological survey results.

In 1989 an outbreak of bluetongue on a sheep farm in Xiangfan, Hubei Province, caused serious economic loss: 1505 (81%) of the 1837 sheep showed disease, and 521 (34%) of these died. From 1990 to 1994, agar gel immunodiffusion (AGID) was used to distinguish seropositive sheep from seronegatives. The seropositive sheep were eliminated. As a preventative measure, the seronegative sheep were immunised with an inactivated vaccine made at the Yunnan Tropical and Subtropical Animal Virus Diseases Laboratory.

The economic loss because of sheep mortality was RMB 86850 (in 1995, 1 Chinese Renminbi (RMB) was equivalent to US$0.123), the cost of medication was RMB 23000, and the loss of breeding animals (982 sheep infected with bluetongue) was valued at RMB 140000.

Since inactivated vaccination was first applied in 1991, the situation has changed substantially. Proven effective by a provisional experiment in 1990, vaccination has been carried out extensively on the Xiangfan breeding farm since 1991. Of the total 3195 sheep immunised with inactivated vaccine up until 1994, only 18 animals (0.6%) have been found infected by bluetongue virus (BLU), and none were found in 1992 and 1994. Morbidity has declined. Over the four-year period, bluetongue disease occurred in only 18/2939 (0.6%) and no sheep died. In 1991, morbidity was 11/1012 (1.1%) with no deaths. In 1992, 609 sheep were vaccinated and there was no bluetongue on the farm that year. In 1993, morbidity was 7/546 (1.3%) with no deaths, compared to 178 (39.3%) of 452 unvaccinated controls that developed bluetongue, 31 (17.4%) of which died. In 1994 804 sheep were vaccinated and there was no bluetongue on the farm that year. The AGID positive rate has also declined, from 81% in 1989 to 13.9% (505/3624) from 1991 to 1994. These results demonstrate that bluetongue has been effectively controlled.

Together with inactivated vaccine, other comprehensive controls were used, including serological monitoring by AGID testing; strict quarantine when

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introducing breeding sheep; and eliminating vector insects such as *Culicoides*.

As a result of the vaccination program and other measures, from 1991 to 1994 economic losses decreased significantly: 2459 breeding sheep did not have to be rejected for being seropositive (value RMB 491800), 521 sheep were saved by vaccination (value RMB 156300) and there was a saving in the cost of medication that would have been needed for bluetongue therapy (RMB 183000). This gives an estimate of RMB 831100 saved through the vaccination program.

In summary, the inactivated bluetongue vaccine was extremely effective on the Xiangfan breeding farm, as reflected both in epidemiological and economic analysis.

Development of Bluetongue Attenuated Vaccine in China

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Abstract

Previous studies elsewhere have shown that an attenuated vaccine for bluetongue, produced through passages in embryonated chick embryos (ECE), could protect sheep successfully. In China, we have also developed this kind of vaccine, using virulent strains of bluetongue virus isolated from clinically ill sheep in Yunnan and Sichuan Provinces and that differ from one another in neutralisation tests. These viruses were passaged in sheep two to three times and remained virulent. Blood from these sheep were serially passaged through ECE to produce the crude vaccines. There were some differences in the development procedures used to make vaccines from the Yunnan and Sichuan strains. In an immunisation experiment with the Sichuan strain, sheep were divided into two groups, with an additional control group. One group was immunised with Sichuan ECE vaccine. Another group was immunised first with the Yunnan ECE-vaccine and then with the Sichuan ECE vaccine. Both groups were then challenged with the highly virulent Sichuan strain. From this experiment, we confirmed the attenuation of the virulence of the Yunnan and Sichuan strains by the ECE passages, although some subclinical symptoms (such as elevated temperature and changes in white blood cells) occurred in a few sheep. In these immunisation experiments, the ECE vaccines were shown to be effective, with greatest effectiveness after 47 passages ($ID_{50}=10^{3.6}$) and reduced effectiveness after 60 passages. From 14 to 21 days after vaccination, 90% of sheep could be protected when challenged by highly virulent strains.

SINCE the beginning of this century, the development and production of bluetongue vaccines has been studied, first in South Africa and later in the USA. The earliest vaccination involved inoculating blood, containing virulent virus, from a sick sheep, plus the serum of recovered sheep. In South Africa, Spruell (1906) followed the procedure for producing attenuated *Theileria* vaccine by passaging in sheep ten times (Theiler 1908). This method was used effectively for more than 40 years (Neitz 1948). However, chick embryo vaccine was substituted when Alexander et al. (1947) showed that virulent BLU could be attenuated via passaging in embryonated chick embryos (ECE) when inoculated through either vein or amnion and incubated at 33.5–34°C. A multivalent vaccine was subsequently developed by Alexander and Haig (1951). In the USA, McKercher et al. (1957) developed an ECE-attenuated vaccine using local isolates. Later research indicated that ECE vaccine did not return to virulence after several passages in sheep, and sheep inoculated with virulent BLU were not susceptible six weeks after vaccination with ECE-attenuated vaccine, with a protection rate of 92% (McGowan et al. 1956). Transmission via *Culicoides* was always a possibility (Foster et al. 1968). Other BLU vaccines have been developed since then via tissue culture of ECE-adapted virus (Kemeny and Drehle 1961). Commercial vaccine for bluetongue is now available in South Africa and USA.

In 1979, the economic loss attributable to bluetongue outbreaks in ruminants in China was significant because of the lack of a vaccine (Zhang Nianzu et al. 1989). The work on developing vaccines and vaccination procedures commenced in 1980. This paper reports development of an ECE-attenuated vac-

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cine in the epidemic areas of the Provinces of Yunnan (begun in 1980) and Sichuan (begun in 1988). Two isolates from these areas were identified as significant serotypes in China.

Materials and Methods

Original isolates as inocula

Yunnan inocula Y33-F2-89, Y55-F3-447 and Y55-F3-471 were passaged two to three times after isolation from naturally infected sheep no. 33 (Y33) and no. 55 (Y55). The storage period of all inocula was less than 30 days.

Sichuan inoculum SW-0.926 (isolated from naturally infected sheep blood; Lin Lihui et al. 1989) was passaged in sheep two to three times, frozen and thawed three times at –80°C, then lysed by ultrasonication, just before use.

Eleven-day-old ECE were inoculated intravenously by the methods of Goldsmit and Barzaili (1968) and Foster and Luedke (1968), and amnion inoculated using seven- to eight-day-old ECE. The eggs were provided by a specific pathogen-free breeding farm. The amounts for inoculation were as follows: 0.2 mL were used for amnion or inoculation and 0.02 mL for vein; five eggs per inoculum; 5–15 eggs per passage. These were incubated at 35°C for 7 days, and discarded if ECE died within 24 hours. Dead embryos were stored at 4°C, while all live embryos were chilled at 4°C. At harvest, a 1:5 homogenate of the ECE muscle and liver was made in saline (pH7.2), containing 100 IU penicillin and streptomycin per mL. The product was frozen and thawed twice, then stored at 4°C for 48 hours before use. Five to fifteen ECE were used per inoculum at each passage level, using the above procedure. Batches with less than 50% mortality in the previous three to five passage were repeated.

The virulence was measured by following the protocol of Zhang Khaili et al. (1991). Sheep were inoculated with 5–10 mL homogenate of ECE Y33-89F4, 6, 8, 25, 47 and 108; and Y55-471F13 and 22; Y55-471F46 and 53 in Yunnan, and F26, 30 and 35 in Sichuan. Immunofluorescence of ECE organs, including heart, liver, brain, amnion and its fluid and the body (Y33-89F52; Y55-447F57, 65, 74 and 75; and Y55-471F46, 53), was tested on frozen sections (Hu Yuling et al. 1989). AGID tests were performed by standard protocols (Anon. 1989).

Two sheep per specimen were inoculated, with 4 mL per sheep of the ECE homogenate proved to be attenuated, including Y33-89F51 and F115 (Yunnan). Blood was collected seven days post-infection, the serum discarded and the red cells resuspended in saline, then blind passaged in sheep four times. The sheep were observed for clinical signs. The original experimental sheep were challenged at 14–21 days post infection with 100 IC_{50} of virulent virus. The LD_{50} of per passage of homogenate of ECE was calculated.

Vaccination experiment

The vaccination and challenge experimental procedure is summarised in Tables 1 and 2. All sheep were Xinjiang Merinos which tested negative for bluetongue antibodies before vaccination (Hu Yuling 1991). Two or three healthy sheep were used as controls. Body temperature and clinical signs were recorded daily. Leucocyte numbers were counted every two days.

Healthy sheep were inoculated with one of several dilutions of the attenuated inocula from ECE of Y33-89F47, 88, 51 and 59. Challenge was with one of the virulent blood inocula of Y33F3-778 and 505 and Y33F16-459 at 14, 21 and 35 days post-infection respectively. The body temperature, leucocyte numbers and clinical signs were compared with the control group to calculate the vaccination efficiency.

Three sheep were vaccinated with 5 mL of Y55-471F36 ECE homogenate, and blood was collected at 5, 8, 14, 30, 90 and 120 days post-infection. The virus was separated from the blood sample (following the protocol of Zhang Khaili et al. 1991) and the ECE inoculated into the amnion: these were then blind passaged five times. A positive result was indicated when 50% of ECE died in three successive passages within five days of inoculation.

Table 1. Vaccination procedure for bluetongue in Yunnan Province.

Vaccination inoculum (from ECE)	No. of sheep	Volume of inoculum (mL)	Challenge (days post-infection)	Challenge inoculum (from blood)	Volume and dose of challenge
Y33-89F46	2	5	35	Y33F-459 or Y33F3-505	0.5 mL (50 ID_{50})
Y33-89F47	3	5	35	Y33F-459 or Y33F3-505	0.5 mL (50 ID_{50})
Y33-89F75	3	5	35	Y33F-459 or Y33F3-505	0.5 mL (50 ID_{50})
Y33-89F108	3	5	35	Y33F-459 or Y33F3-505	0.5 mL (50 ID_{50})
Y55-471F46	3	5	35	Y55-F5-598	3 mL (300 ID_{50})

Table 2. Vaccination procedure for bluetongue in Sichuan Province.

Trial	No. of sheep	Basic inoculum		Second inoculation (42 days post-infection)			Challenge	
		Inoculum (ECE)	Volume and dose	Inoculum (ECE)	Volume and dose	Days post-inoculation	Inoculum (blood)	Volume and dose
1	5	SWF38	2 mL, 1:50	–	–	21	Sichuan virulent	0.5 mL 100 LD_{50}
2	1	Yunnan attenuated vaccine	5 mL, 1:10	SWF22	5 mL, 1:50	14	virulent specimen	0.5 mL, 100 LD_{50}
	1	"	1:50	Treatment for other dosages at 1:10				
	1	"	1:100					
	1	"	1:1000					
	1	"	1:1500					

Results

Development of ECE attenuated vaccine

The results of the inoculation and passaging of virulent blood specimen to ECE are shown in Table 3. ECE death, with swollen and haemorrhagic embryos, occurred regularly after 5–8 passages. Passaging was continued to a maximum of 113 times. The pathogenicity of BLU was attenuated after 46 passages in ECE although it could cause some clinical signs at 5–25 passage when tested in sheep (Table 4).

Table 3. Inoculation and passage in ECE for development of bluetongue vaccine.

Inoculum	Route	Passage no.	Duration of passage (days)	Cycle of passage (average days per passage)	Passage number for recovery of virulence
Y33-89 I	amnion	103	584	5.1	117
Y33-89 II	1–7 passage with vein then to amnion	113	679	5.1	114
Y55-471	amnion	45	427	9.26	89

Table 4. Pathogenicity test of bluetongue virus attenuated via ECE passage.

Inoculum	Dilution of inoculum	Inoculum mL	No. of sheep	Observation (symptoms) Subclinical	No. of sheep Clinical	Pathogenicity ratio
Y55-447F13	–	5	3	1	1	1 : 3
Y55-447F22	–	5	3	1	1	1 : 3
Y55-471F23	–	5	3	1	1	1 : 3
Y55-472F46	–	5	3	0	0	0 : 3
Y33-89F5	–	10	2	2	0	2 : 2
Y33-89F6	–	10	3	3	0	3 : 3
Y33-89F7	–	10	2	2	0	2 : 2
Y33-09F25	–	10	3	3	3	3 : 3
	1 : 5	10	3	0	1	1 : 3
	1 : 10	10	3	3	3	3 : 3
	1 : 20	10	3	0	0	0 : 3
	1 : 200	10	3	3	3	3 : 3
Y33-89F46	–	5	2	0	0	0 : 2
Y33-89F75	–	5	3	0	0	0 : 3

Immunofluorescence was used to test the virulence of the vaccine in different ECE passages and to the organs (Tables 5 and 6 respectively). Positive immunofluorescence occurred in all passages from 8 to 29 (Table 5). Strong evidence of virus was found in heart muscle and liver, but none in the organs of ECE at passage 46, except a slight positive with amnion. Virulence increased with passage number (Table 7). Different organs were titrated (Table 8).

Table 5. Immunofluorescence tests of different passages of ECE of the Sichuan strain.

Date	Passage no.	Results
16 October 1988	F8	+++
26 January 1989	F17	+++
1 March 1989	F20	++++
29 March 1989	F22	++++
6 May 1989	F29	++++

In safety tests, there were no clinical signs observed for 21 days in both experimental sheep inoculated with attenuated vaccine passed four times (Tables 9 and 10, Sichuan and Yunnan respectively). Challenges with virulent blood did not cause any clinical symptoms (Table 10). The two control sheep became sick.

Vaccination experiment

In protection experiments in Yunnan and Sichuan, only one of the experimental sheep had clinical symptoms of bluetongue after challenge (Tables 11 and 12, Yunnan and Sichuan respectively). All the control sheep developed bluetongue after challenge.

Evaluation of vaccine efficiency was conducted in both Yunnan and Sichuan by calculating the titre from the results in Tables 11 and 12, together with the results of inoculating blood taken 5–120 days post-infection which had been blind passaged in ECE five times. No regular ECE death pattern was found.

Table 7. Titration of different passages of chick embryos.

Date	Inoculum	Titration (ID_{50}=Log)	Remarks
2 Jun 1983	Y55-471F50	$10^{5.13}$	0.2 mL inoculated to amnion, inoculum prepared with whole body of embryo
12 Jul 1983	Y55-471F64	$10^{4.34}$	
11 Aug 1983	Y55-471F47	$10^{4.33}$	
29 Mar 1987	Y55-471F44	$10^{3.83}$	
29 Mar 1987	Y55-471F27	$10^{3.83}$	

Discussion

ECE-attenuated vaccines, developed from local isolates from sheep sick with bluetongue in Yunnan and Sichuan, vaccinated sheep effectively (Table 4). Observation of experimental sheep showed no clinical signs on sheep at 21 days post-infection, as also found by McGowan et al. (1956).

The procedure for developing and evaluating a vaccine was established and proven in both provinces.

Table 6. Immunofluorescence tests of different passages of ECE of the Yunnan strain.

Inoculum via amnion		Organs of embryos			
		Muscle	Liver	Heart	Brain
Y33-89	F52*	–	–	–	–
	F54	+	++	+–	–
	F59	–	++	–	–
	F59 (vein)	–	+++	nt	nt
	F46	#	#	+++	+
Y55-447	F53*	–	–	–	–
	F57	++	++	–	–
	F65	++	++	–	–
	F74	+	++	+	–
	F75	+++	++	++	–

nt not tested
* discarded
\# inconclusive
– negative
+– trace of fluorescence
+, ++, +++ relative strength of positive fluorescence