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Clinical and pathological changes associated with the propagation of Indonesian Bluetongue viral isolates in Merino sheep

I. Sendow[1], H. Hamid[1], Sukarsih[1], E. Soleha[1], S. Bahri[1], M. Pearce[2] and P.W. Daniels[3]

[1] Research Institute for Veterinary Science. PO Box 52, Bogor, Indonesia
[2] Indonesia International Animal Science Research and Development Foundation (INI ANSREDEF), Bogor, Indonesia
[3] CSIRO Australian Animal Health Laboratory, PO Bag 24 Geelong, 3213, Australia.

Abstract

Nine Merino sheep imported from Australia and seronegative for antibodies to bluetongue viruses were inoculated intravenously or subcutaneously with cattle blood from which bluetongue virus (BTV) serotypes 1, 9, 21 or 23 had been isolated. Seven Merino sheep were inoculated with uninfected cattle blood as placebo. Seroconversion occurred in 5 sheep inoculated with BTV serotypes 1, 9 and 21 and virus was reisolated in embryonated chicken eggs. Embryos died between 3 to 5 days post inoculation (PI) and were dwarfed and haemorrhagic. Passaging in BHK-21 cells produced cythopathic effect. These infected cells and dead embryos reacted in a monoclonal antibody based BTV antigen capture ELISA. Pyrexia occurred in 7 sheep inoculated with BTV serotypes 1, 9 and 21 between 6 and 11 days PI, and very mild to moderate clinical signs were produced between 7 and 14 days PI. Hyperaemia of the buccal mucosa and coronitis were the most common signs in infected sheep. No sheep died or was severely affected during these experiments.

Key words: Symptoms, pathological changes, isolation, bluetongue virus.

Introduction

Bluetongue is an insect transmitted, non-contagious viral disease of sheep caused by bluetongue viruses (BTV), members of the family Reoviridae, genus Orbivirus (Gorman *et al.*, 1992). BTV infections are common in ruminants throughout tropical and subtropical areas of the world. However, bluetongue disease primarily affects sheep (Erasmus, 1975). BTV infections of cattle are asymptomatic (MacLachlan *et al.*, 1992), and cattle are considered to be a natural reservoir host for BTV (Erasmus, 1975; MacLachlan *et al.*, 1992). In many countries indigenous sheep are reported

to be less susceptible than European breeds such as Merinos (Erasmus, 1975). Clinical signs of BTV infection in sheep include pyrexia; hyperaemia of buccal and nasal mucosae; inflammation, ulceration and oedema of the oral mucosa; salivation and frothing at the mouth, with the latter associated with persistent licking movements of the tongue. Nasal discharge varies in degree from watery to mucopurulent or blood stained, and dries to form crusts. Lachrymation and hyperaemia of the eyelids and ocular mucous membranes are often observed. Oedema of lips, tongue, intermandibular space, neck, ear and at the base of the horns may be observed. In severe cases, the tongue may become hyperaemic then markedly swollen and oedematous, then cyanotic (Erasmus, 1975; Pini, 1976; Gard, 1987).

Although mortalities from bluetongue disease can be up to 30% (Erasmus, 1975), the manifestations of infection can be extremely variable, and inapparent infections are usual in many countries.

In Indonesia, antibodies to BTV have been detected in ruminants with prevalences varying with species, breed, serotype of BTV and geographical location (Sendow *et al.*, 1986, 1991a). Eight serotypes of BTV have been reported in Indonesia, serotypes 1, 3, 7, 9, 12, 16, 21 and 23 (Sendow *et al.*, 1991b; 1993 a,b; 1996), isolated from healthy sentinel cattle blood. Clinical signs of bluetongue have not been reported in local ruminants. However an outbreak in imported Suffolk sheep consistent with bluetongue disease was observed in 1981 (Sudana and Malole, 1982).

The objectives of this study were to obtain preliminary indications of virulence of Indonesian BTV isolates under experimental conditions in Merino sheep, and to propagate local isolates in these presumed susceptible sheep.

Materials and Methods

1. Viruses

Bovine blood samples from which BTV serotypes 1, 9, 21 or 23 had been isolated were used in these experiments. Aliquots of Friesian Holstein (*Bos taurus*) blood collected in West Java and from which BTV serotypes 1 or 9 had been isolated (Sendow *et al.*, 1992; Sendow *et al.*, 1993a) and aliquots of Bali cattle (*Bos javanicus*) blood from Jayapura, Irian Jaya, from which BTV serotypes 21 or 23 had been isolated (Sendow *et al.*, 1993b) were retrieved from liquid nitrogen and thawed immediately prior to use. Due to the small volumes of stored blood available, titration of virus in blood samples was not performed before inoculation.

2. Sheep

Merino sheep more than 4 years old were imported from Australia. The sheep were from the same source and of the same genetic strain as those used in bluetongue viral pathogenicity experiments in that country (Johnson *et al.*, 1992). All sheep were free of detectable antibody against BTV as assessed by competitive-ELISA (C-ELISA, Lunt *et al.*, 1988). The tem-

perature, the mucosae of eyes, nose and mouth, and the feet were checked before the experiment.

3. Experimental procedures

Pairs of Merino sheep were each inoculated intravenously and subcutaneously with total volumes of 0.7 ml to 1.3 ml of the blood samples. Other pairs of Merino sheep were each inoculated with uninfected cattle blood, as placebo. Infected and control sheep were kept in insect proof accommodation. Sheep were examined for clinical signs daily for 4 weeks. Serum and heparinized blood were collected prior to inoculation, 1 day post inoculation (PI), 7 days PI or when the temperature rose to more than 39.8°C, and at 28 days PI. Sera collected at 28 days PI were tested for BTV antibodies using the C-ELISA. Sheep were killed and necropsied at the end of observation period (28 days PI).

4. Viral isolation

Heparinized blood samples collected at 7 days PI or when the temperature had risen to more than 39.8°C were inoculated intravenously into five 11-day-old embryonated chicken eggs (ECE). ECE were incubated at 33.5°C for 5 days, and observed daily for embryo death. Embryos dying on the first day PI were discarded. Embryos dying on the second to the fifth days PI were homogenized in phosphate buffered saline (PBS) and inoculated into *Aedes albopictus* cell cultures before passaging into BHK-21 cell cultures. Infected cell cultures that showed cythopathic effect (CPE) were tested for BTV using an antigen capture ELISA (Ag-C-ELISA) test.

5. Serological testing

Sera were tested for antibodies to BTV in a commercial C-ELISA kit (*Trop-Bio*, Townsville, Australia). Sera with an inhibition level of more than 40% were considered to be BTV reactors.

Results

1. Propagation of virus in sheep

Not all nine inoculated Merino sheep showed clinical signs or seroconversions (Tables 1 and 2). All sheep inoculated with BTV serotypes 1 and 21 seroconverted, but only 1 of 3 sheep inoculated with serotype 9 supported viral replication. The sample of serotype 23 failed to infect inoculated sheep.

2. Viral Assay

Infected sheep blood was assayed for BTV. The results are shown in Table 1. Titres in inoculated sheep varied from 102.3 to 104.4.

3. Clinical observations

i. Pyrexia

The first clinical sign of disease in sheep responding to inoculation was a rise in temperature. Seven of 9 inoculated sheep showed a febrile peak between 39.8°C and 40.5°C, which occurred between 6 to 11 days PI , as shown in Table 2.

ii. Oral and nasal lesions

The most common clinical sign was erythema of the oral and buccal mu-

cosae. This varied from mild pink to intense purplish red (Figure 1). Buccal erythema was occasionally followed by petechial haemorrhages on the lip and gums and sometimes by ulceration or erosion.

iii. Ocular lesions
Conjunctivitis developed in five sheep inoculated with BTV serotypes 1, 9 and 21.

iv. Oedema
Oedema of eyelids, lips and nose were observed in six sheep between 7 to 14 days PI.

v. Foot lesions
A mild to moderate coronitis developed in seven sheep inoculated with BTV type 1, 9 and 21, but affected sheep still could stand and walk . Only two sheep (No. 1244 and 0084) showed a distinct red band in the periople tissue just above the coronet. Mild inflammation of the posterior hooves was observed at 7 to 9 days PI. This persisted for one week before the colour changed to become cyanotic. The anterior and the posterior aspects of the hooves of most feet were affected. No clinical signs was produced in sheep inoculated with BTV serotype 23.

4. Gross pathology

At necropsy at 28 days PI, haemorrhages in the pulmonary artery were found in only one infected sheep. Dilatation of the heart was observed in seven infected sheep. Froth in the trachea was also found in two sheep while pulmonary congestion was found in all sheep.

Table 1. Routes of inoculation, seroconvertion and titre of sheep blood inoculated in merino sheep against Indonesian Bluetongue isolates.

Animal #	BTV	Source	Routes	Seroconvertion	Titre of sheep blood after inoculation
0037	1	Cattle blood	iv, sc	+	103.2
1244	1	Cattle blood	iv	+	103.8
0126	9	Cattle blood	sc	-	-
005	9	Cattle blood	sc, iv	-	-
BP8	9	Cattle blood	iv	+	102.3
BP1	21	Cattle blood	iv	+	103.8
0084	21	Cattle blood	sc	+	104.4
0093	23	Cattle blood	sc	-	-
0038	23	Cattle blood	iv	-	-

iv = intravena
sc = subcutaneous

Table 2. Clinical signs produced by inoculation of Bluetongue viruses in merino sheep.

Animal #	BTV	Fever	Hyperaemia in the mouth	Hyperaemia in the eyes	Lips oedema	Inflamation/ coronitis	Nasal discharge salivation
0037	1	+, 10-14 dpi	+, 10-14 dpi	+, 10-14 dpi (discharge)	+, 10-12 dpi	+, inflamation on posterior of the feet, 9-13 dpi	+, 7 dpi
1244	1	+, 8-10 dpi	+, 7-9 dpi	+, 10 dpi	+, 8-11 dpi	+, coronitis, 9-15 dpi	+, 4 dpi
0126	9	+, 7dpi	+, 8-10 dpi	+, 8-11 dpi	+, 8-10 dpi	+, inflamation on posterior of the feet, 7 dpi	-
005	9	+, 7-8 dpi	-	-	-	±, not	+, 20 dpi
BP8	9	+, 10-11 dpi	+, 7-16 dpi	-	+, 7 dpi	+, inflamation on posterior of the feet, 7-13 dpi	+, 20 dpi
BP1	21	+, 6-10 dpi	+, 7dpi	+, 10 dpi	+, 7-9 dpi	+, inflamation on posterior of the feet, 9-14 dpi	-
0084	21	+, 7-9 dpi	+, 7-11 dpi ulcus at 9 dpi	+, 9-12 dpi	+, 7-10 dpi	+, coronitis, 7-14 dpi	-
0093	23	-	-	-	-	-	-
0038	23	-	-	-	-	-	-

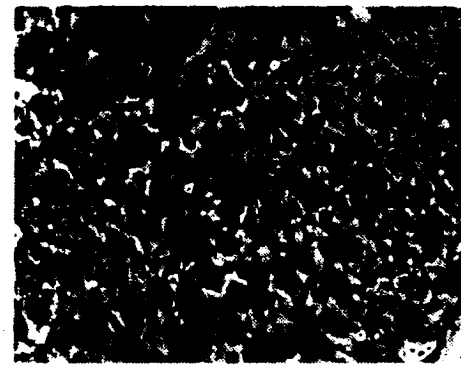
dpi = day post infection

5. Histopathology

i. Heart
Haemorrhage in the myocardium and focal myocarditis were found in four infected sheep (BP8, 0126, 1244 and 0037). There was also severe degeneration of myocites, phagocytosis of necrotic cells, and some fibrosis and infiltration of lymphocytes and macrophages in the interstitial tissue (Figure 1). Myocardial arteries showed marked medial thickening, splitting of elastic lamina and intimal proliferation.

ii. Lung
Moderate to marked multifocal congestion and haemorrhages with alveolar oedema and interlobular oedema of variable severity were found in all control and infected sheep. Mononuclear cell infiltration of alveolar septae was present in four inoculated sheep and two control sheep.

Figure 1. Focal interstitial inflamatory cell infiltration and degeneration of myocites (H&E 380.1 X)



Discussion

BTV infections are widely distributed in Indonesia (Sendow *et al.*, 1986, 1991a), and eight serotypes of BTV have been isolated (Sendow *et al.*, 1996), three from locations some 4000 Km apart (Sendow *et al.*, 1993b). Hence it is important to understand more of the biology of these viruses, especially their pathogenicity for sheep. In spite of serological evidence of infection, local sheep have not shown bluetongue disease, even where intensively monitored in research of traditional village husbandry systems (Adjid and Daniels, 1993). However, imported Suffolk sheep have shown clinical disease consistent with BTV when introduced into the same area in Indonesia (Sudana and Malole, 1982). This suggests that some Indonesian strains of BTV may be pathogenic in susceptible animals, an aspect which the present study begins to address.

Gard (1987) demonstrated that BTV passaged in cell culture loose virulence. Hence, pathogenicity studies must be conducted with non-cell culture adapted viruses, preferably viruses maintained in passage among sheep and cattle. To ensure that such viruses would be available for subsequent studies, the collection protocols for viral isolation have always included splitting of the heparinized blood samples and storage of at least one aliquot in liquid nitrogen .

(Daniels *et al.*, 1991; Sendow *et al.*, 1992).

Since the ability of local BTVs to propagate at high titre in Indonesian sheep may be in doubt because the mechanism of their reduced susceptibility to disease is not known, it was considered most prudent to multiply the BTV isolates in naive sheep of known susceptibility. In Australia, Merino sheep from the Toorak Research Station of the Queensland Department of Primary Industries have been shown to be susceptible in pathogenicity experiments (Johnson *et al.*, 1992), so animals were obtained from the same source to use in these experiments.

Sheep inoculation is considered one of the more sensitive tests for the presence of BTV under international protocols (Afshar and Gard, 1992). The failure of some inoculated sheep to seroconvert in this study may indicate that not all stored samples contained viable virus, although again individual animal variation has been observed where some sheep inoculated with viable virus have not responded (Melville, pers. comm.). The titres of viraemia measured in this study indicate that the experiment has yielded stocks of BTV serotypes 1, 9 and 21 suitable for more detailed studies of their virulence.

The clinical responses of the sheep in the current experiment can be best classified as mild. Apart from moderate fever, the most consistent clinical signs were hyperaemia of the oral mucosa and coronitis. In other similar experiments inflammation of the oral and nasal mucosa has also been a consistent finding (Gard, 1987).

However, the severity of clinical response was considerably milder than that of classical bluetongue disease, as reviewed by Erasmus (1975). Although there can be marked individual animal variation in bluetongue disease, fever can be 41°C, for an average 6 to 7 days. In more severe cases of classical bluetongue, an increased respiratory rate is markedly obvious, hyperaemia of the oral and nasal mucosa is accompanied by frothing at the mouth and persistent licking movements of the tongue, a nasal discharge becomes mucopurulent and forms crusts on the nostrils, and there is conjunctivitis. These signs progress to oedema of the lips, tongue and lower jaw. Petechial haemorrhages occur in the mouth. The tongue may be hyperaemic or cyanotic, and hence blue in color, or yellow in places and loosing epithelium. Epithelium elsewhere in the mouth may also excoriate, leading to bleeding. Foot lesions develop later in the disease, the feet becoming hot and painful with reddening of the coronary band. Such sheep are very lame. Muscle degeneration may also contribute to sheep becoming moribund. Mortalities in severe outbreaks are up to 30%.

In the current experiments only one sheep (BP8) showed focal haemorrhages at the base of the pulmonary artery. This lesion was considered to be pathognomonic of BTV infection (Erasmus, 1975). This sheep was infected with BTV serotype 9. Sheep were not necropsied during clinical illness, but at day 28 PI, some 1 to 2 weeks after the regression of clinical signs. Hence minor pathological changes

could have resolved. Most of the pathological changes recorded were considered non-specific.

No animal died in these experiments, a different situation from the field outbreak of bluetongue disease in Suffolk sheep, where mortality was reported to be high (Sudana and Malole, 1982). It has been noted in other countries that there is variability in breed susceptibility and among individual sheep. This variability may also be associated with physical condition of the animals, serotype or strain of the virus, age of animals, concurrent disease status at the time of infection or the virulence of the BTV involved in the outbreak (Parsonson, 1992). Further pathogenesis studies on BTV in local and imported sheep are recommended to gain clearer information. Only a small number of strains of Indonesian BTV have been examined to date.

The observation of low virulence may not be attributable to serotype *per se*. Although BTV serotypes 1 and 9 from South Africa are usually ranked as highly pathogenic (Gard, 1987), comparison of Australian and South African serotype 1 has shown the Australian strain to be much less virulent (Hooper *et al.*, 1996). In the current work BTV serotype 1 was only mildly virulent. Similarly, an Australian isolate of BTV serotype 9 has been found to be virtually avirulent in one study (Johnson *et al.*, 1992). While perhaps associated with the most severe pathology as shown by the haemorrhage at the base of the pulmonary artery in the current study, this Indonesian BTV type 9 induced only mild or non-specific clinical signs and was certainly not as highly virulent as the serotype 9 in South Africa.

It is unfortunately that the Indonesian isolate of BTV serotype 23 tested on this occasion did not replicate, as this serotype has been shown in one Australian study to be moderately to severely virulent (Johnson *et al.*, 1992).

The current work has provided stocks of wild type virus for these Indonesian serotypes, suitable for further investigations of the virulence of these viral strains and for investigations of the comparative susceptibility of local breeds of Indonesian sheep. Only when such information is complete for a representative sample of the full range of Indonesian BTV isolates will the epidemiology of bluetongue in Indonesia be clearer, and the relative risks of importing susceptible sheep from other countries more clearly understood.

Abstrak

Gejala klinis dan perubahan patologi setelah infeksi isolat Bluetongue pada domba merino

Sembilan domba merino yang diimpor dari Australia dan tidak mempunyai antibodi terhadap virus Bluetongue (BTV) telah diinokulasi secara *intravena* dan *subcutan* dengan darah sapi dimana virus BTV tipe 1, 9, 21 atau 23 telah berhasil diisolasi. Tujuh domba merino juga diinokulasi dengan darah sapi normal sebagai kontrol. Serokonversi terjadi pada 5 domba yang diinokulasi dengan virus BT tipe 1, 9

dan 21 dan virus dapat diisolasi kembali pada telur embrio tertunas. Embrio mati pada hari ke 3 hingga 5 setelah inokulasi, perdarahan dan kerdil. Pasase pada biakan jaringan BHK-21 menimbulkan *cytopathic effect* (cpe). Biakan jaringan dan suspensi embrio tersebut bereaksi dengan monoklonal antibodi BTV pada uji antigen capture ELISA. Demam ditemukan pada 7 domba yang diinokulasi oleh BTV seotipe 1, 9 dan 21 antara hari ke 6 dan 11 setelah inokulasi, dan gejala klinis dengan derajat yang sangat ringan hingga sedang terjadi pada hari ke 7 hingga 14 setelah inokulasi pendarahan pada mukosa mulut dan peradangan coroner sangat sering ditemukan pada domba yang terinfeksi. Tidak ada satupun domba yang mati ataupun sakit parah selama percobaan ini.

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