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Isolation of bluetongue virus serotype 21 from *Culicoides* spp. in Indonesia

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ABSTRACT

The isolation of a bluetongue (BLU) virus from *Culicoides* spp. in Indonesia is reported. BLU serotype 21 was isolated from a mixed pool of *C. fulvus* and *C. orientalis* of the *Avaritia* subgenus.

INTRODUCTION

Bluetongue (BLU) virus is an arbovirus infecting ruminants and causing disease in sheep (Erasmus, 1975). In Indonesia, clinical bluetongue disease has been reported in imported but not in local sheep (Sudana and Malole, 1982). Antibodies to BLU and related orbiviruses have been reported (Sendow et al., 1986; Sendow et al., 1991a), BLU viral serotypes 7 and 9 have been isolated from sentinel cattle in West Java (Sendow et al., 1991b), but there is no information on BLU vectors in Indonesia. This paper reports the isolation of a BLU virus from *Culicoides* spp.

MATERIALS AND METHODS

Insect collection

Insects were collected at a sentinel cattle site at Depok (6°24′S, 106°48′E) in West Java, at an altitude of 95 m, with an average annual rainfall of 2500 to 3000 mm and an average temperature of 27°C. This farm had about 30 cattle. Faeces were heaped close to the pens for periods of several weeks prior to spreading on adjacent fields. Drainage was poor. Previous light trap collections of insects had shown *Culicoides* to be abundant (Sukarsih unpub-

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lished), and BLU viruses had been isolated from blood of the sentinel cattle (Sendow et al., 1991b).

Insects were collected with a Pirbright-type miniature light trap operated from the mains supply via a 12 volt step-down transformer. Collections were made from 4.30 pm to 8.00 pm once a week from February 1991 to January 1992. In the trap, insects were blown directly into a bottle containing phosphate buffered saline (PBS) supplemented with 1000 μg/ml kanamycin, 500 mg/ml streptomycin and 500 IU/ml penicillin with 0.1% household detergent. The collections were stored overnight at 4°C and sorted next morning using a binocular microscope. *Culicoides* spp. were separated and pooled into groups of the subgenus *Avaritia*, the subgenus *Hoffmania* and subgenus *Trithecoides*, with separate pools for *C. oxystoma* and *C. actoni*. Only non-engorged females were selected for viral isolation.

Viral isolation

Pools of non-engorged females containing up to 250 insects were ground in sterile glass tissue grinders containing 2.5 ml PBS with antibiotics (100 mg/ml streptomycin, 100 IU/ml penicillin and 100 μg/ml kanamycin). The resulting suspensions were clarified by centrifugation at 1000 g for 10 min. A volume of 0.1 ml of each supernatant fluid from each pool was inoculated intravenously into each of four 11-day-old embryonated chicken eggs (ECE). Embryos dying on the first day post inoculation were discarded. The heart was dissected from embryos which died from the second to the fifth day. Hearts from replicate ECE were pooled and ground in 2 ml vols of Eagles minimum essential medium (Flow Laboratories) containing 2% foetal bovine serum (FBS) with antibiotics. Suspensions of heart tissue were clarified by centrifugation at 1000 g for 10 min. The supernatant fluids were filtered through 450 nm filters (Sartorius) before passaging sequentially in *Aedes albopictus* (C6/36) and BHK-21 cell cultures in roller tubes. Inoculation of C6/36 cells with BLU viruses does not produce cytopathic effect (CPE), so these were incubated for one week at room temperature without observation. The cultures were then harvested and sonicated at 20 Hz for 10 s twice before passaging into BHK-21 cells. The presence of viruses was detected by CPE in the BHK-21 cell cultures. Three blind passages were performed before these cultures were considered not to have shown CPE. Samples producing CPE were considered viral isolates.

Viral identification

Preliminary identification of viral isolates was carried out by the immunodotblotting (IDB) test using a monoclonal antibody (mAb) 20E9/B7/G2 supplied from the Australian Animal Health Laboratory (AAHL), Geelong, Australia. This mAb was considered specific for the BLU group of viruses and was not cross reactive with other Orbiviruses (Lunt et al., 1988).

The test procedure was a modification of that described by Afshar et al. (1987). Infected cell culture fluids were sonicated at 20 amplitudes for 10 s. Two 2 μl aliquots of sonicated fluid were spotted onto nitrocellulose paper (NC) (Trans-blot No. 162-0115, Bio-rad Laboratories, Richmond, USA) with a pore size of 0.45 μm, and dried at 37°C for 5 to 10 min. Hydrophobic sites were blocked by immersing the NC in a buffer of 2% skim milk (Difco) in PBS pH 7.5 and rocking at room temperature for 30 min. This blocking solution was replaced briefly by PBS containing 0.05% Tween 20 (PBS-T) to rinse the NC, then by the mAb diluted 1:400 in PBS-T. Reaction of the mAb with membrane-bound viral antigens was facilitated by gentle agitation for 30 min at room temperature. The mAb solution was then removed and the NC washed three times, each for 3 min, with PBS-T. Next, anti-mouse IgG conjugated with horse radish peroxidase (Dakopatts Cat No. P. 260, Denmark) was diluted at 1:250 in PBS-T and rocked gently with the NC for 30 minutes at room temperature. The NC was then washed again as above with PBS-T 3 times. For a peroxidase substrate, 0.05 g of 3,3′-diamino benzidine tetrahydrochloride (DAB) was dissolved in 100 ml of PBS. Two hundred μl of 30% H_2O_2 were added to this substrate before immersion of the NC. The reaction was stopped after 3 minutes by washing the NC with running tap water. Isolates reacting in the IDB test were sent to Onderstepoort Laboratory, South Africa, for confirmation as BLU viruses and for serotyping.

RESULTS

Viral isolation was attempted from 285 pools of non-engorged female insects from February 1991 to January 1992. Pools of the *Hoffmania* subgenus contained at various times *C. peregrinus, C. sumatrae* and *C. insignipennis.* Pools of *Avaritia* contained variously *C. maculatus, C. fulvus, C. orientalis, C. brevitarsis, C. wadai* and *C. jacobsoni.* Pools of *Trithecoides* contained mixtures of *C. barnetti, C. parahumeralis, C. palpifer, C. albibases, C. anophelis* and *C. gewertzi.*

One pool from each of the *Avaritia* subgenus, *Trithecoides* subgenus and *C. actoni* specimens produced haemorrhagic dead embryos in ECE and CPE in BHK-21 cell cultures. One of these isolates, from a pool of the *Avaritia* subgenus collected on 11 May 1991, reacted in the IDB test with the BLU mAb. Serotyping confirmed this as BLU serotype 21. The pool that yielded the BLU serotype 21 isolate comprised 227 *C. fulvus* and 20 *C. orientalis.*

DISCUSSION

The current study was to provide preliminary identification of the groups of *Culicoides* spp. that may function as vectors of BLU viruses in Indonesia. The *Avaritia* subgenus includes known vectors in Australia (*C. fulvus, C. ac-*

toni, C. wadai, and *C. brevitarsis*) and in Africa (*C. imicola*) (Standfast et al., 1985; Wirth and Dyce, 1985). *C. actoni* was pooled separately because of its abundance. The *Hoffmania* subgenus includes *C. insignis* which is known as a BLU vector in Central America and the Caribbean (Greiner et al., 1985). In South-east Asia the group includes *C. peregrinus* which is associated with domestic ruminants and which supports growth of BLU serotypes 1 and 20 after experimental feeding on infected sheep (Standfast et al., 1985). *C. oxystoma* of the Schultzei group was abundant in the area and has been suspected as a potential vector (Standfast et al., 1985).

Pools of the subgenus *Trithecoides* were included, because they were abundant in previous collections from this area (Sukarsih, unpublished). Members of the subgenus *Monoculicoides,* which includes the major North American vector *C. variipennis*, have not been identified in Indonesia (Wirth and Hubert, 1989). Isolations from insects identify those carrying wild type virus, but controlled studies are necessary to confirm that such species support a cycle of infection between natural hosts (Dyce, 1989).

The isolation of a BLU virus from an *Avaritia* pool comprising 90% *C. fulvus* and 10% *C. orientalis* is consistent with information from northern Australia. BLU serotype 1 has been isolated from trapped *C. fulvus* in northern Australia, and *C. fulvus* has been shown to be an efficient vector of BLU serotypes 1 and 20 in sheep to sheep transmission studies. *C. fulvus* has been infected with BLU serotype 21 in the laboratory by feeding on infected sheep (Standfast et al., 1985). The extent to which *C. orientalis* is associated with livestock is not yet clear, but it is noted as a potential BLU vector (Gard and Melville, 1989).

BLU serotype 21 has been isolated previously from sentinel cattle from this site in West Java and also from Irian Jaya (Sendow et al., unpublished), and so is widely distributed in Indonesia. It is also one of the two BLU serotypes, 1 and 21, distributed in Australia beyond the confines of the far north, extending down the east coast throughout the range of the vector *C. brevitarsis* (Gard and Melville, 1989). Its isolation from *Culicoides* spp. in Indonesia contributes to the description of the epidemiology of BLU viruses in Australasia.

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