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Preliminary Characterization of D'Aguilar Virus and Three Palyam Group Viruses New to Australia

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Abstract

Between 1974 and 1980, 424 viruses were isolated at the Long Pocket Laboratories of the Division of Animal Health, CSIRO, either from insects or from the blood of sentinel cattle, and of these, 165 cross-reacted with D'Aguilar virus (an Australian Palyam group virus) in a complement fixation test. Neutralization tests were used to classify these viruses into four serotypes with the isolates D'Aguilar B8112, CSIRO 11, CSIRO 58 and CSIRO 82 as the type strains. The latter three were new to Australia. Like other orbiviruses, these four serotypes were partially sensitive to treatment with ether or chloroform. Neutralizing antibodies against D'Aguilar, CSIRO 11 and CSIRO 58 viruses were detected in sera from cattle, buffalo, deer and sheep but not in sera from humans, horses, pigs or marsupials. Antibodies against CSIRO 82 virus were detected in 85% of 26 buffalo, and 0·4% of 495 cattle sera tested. The antibody distribution in Australia for D'Aguilar, CSIRO 11 and CSIRO 58 viruses fell within the distribution limits of *Culicoides brevitarsis*, the insect from which these viruses were most commonly recovered. The antibody distribution for CSIRO 82 virus, which was isolated from a pool containing *C. schultzei* and *C. peregrinus*, fell within the much more restricted distribution limits of these species. None of these viruses has been associated with disease.

Introduction

D'Aguilar virus (B8112) was first isolated in Australia in 1968 by the intracerebral (IC) inoculation of day-old mice with homogenates of *Culicoides brevitarsis* collected in Queensland (Doherty *et al.* 1972). Serological studies at the Yale Arbovirus Research Unit showed that this isolate was a member of the Palyam group of arboviruses, named after Palyam virus from India, which was the first member of the group to be described. D'Aguilar virus has since been classified as a member of the Palyam group, together with Abadina, Kasba, Nyabira and Vellore. These viruses were separated on the basis of neutralization tests (Berge 1975; Matthews 1979).

The demonstration of biochemical and morphological similarities with the orbivirus genus has resulted in the Palyam group being included in this genus (Borden *et al.* 1971). Some orbiviruses are known pathogens of man (Kemerovo virus) and of other mammals (epizootic haemorrhagic disease of deer, bluetongue and African horse sickness viruses). One member of the Palyam group (Nyabira virus) has been isolated in Africa from aborted bovine foetuses (Swanepoel and Blackburn 1976), thus indicating possible pathogenicity within this group also. In Australia, antibodies to D'Aguilar virus have been detected in cattle, sheep, and buffalo but not in horses, marsupials, or man (Doherty *et al.* 1972; Doherty 1977).

Viruses isolated at the Long Pocket Laboratories of the Division of Animal Health, Commonwealth Scientific and Industrial Research Organization (CSIRO),

between 1974 and 1976 during attempts to isolate ephemeral fever virus (Standfast and St George, unpublished data) were sent to the Queensland Institute of Medical Research (QIMR) for identification. Several isolates were found to be related to D'Aguilar virus (B8112), by complement fixation; however, differences in growth characteristics of some of the CSIRO isolates and serological studies on sentinel herds suggested to us that the isolates were not a homogeneous group.

This paper demonstrates that the Palyam group viruses isolated so far in Australia can be divided into four distinct serological subgroups including D'Aguilar virus which has been named previously, and three new subgroups for which the type strains are described.

Materials and Methods

Viruses

The 424 virus isolates used in this study were obtained from the CSIRO Long Pocket Laboratory virus collection (Standfast and St George, unpublished data). They were isolated from the blood of sentinel animals, or from insects, either by IC inoculation in suckling mice, or by tissue culture inoculation as described by St George *et al.* (1978). The first isolate of each serotype belonging to the Palyam subgroup has been designated as the type strain, and these were isolated as follows.

CSIRO 11 was isolated by the IC inoculation of suckling mice from a mixed pool of *Culicoides* spp. collected at Beatrice Hill (131·20° E., 12·39° S.) in November 1974 (Standfast and Dyce, unpublished data). CSIRO 58 was originally isolated by inoculating cow blood into baby hamster kidney (BHK21) tissue culture tubes (St George, unpublished data). The blood was collected at Grafton (152·56° E., 29·41° S.) in March 1976. CSIRO 82 was isolated from an insect pool containing *C. schultzei* and *C. peregrinus* collected at Beatrice Hill in October 1975 (Standfast and Dyce, unpublished data) and inoculated IC into suckling mice. The B8112 isolate of D'Aguilar virus (Doherty *et al.* 1972), obtained from the QIMR, was passaged a further four times in BHK21 cells before testing. CSIRO 947 (Reovirus type 2) and Murray Valley encephalitis (MVE) virus were used as positive and negative controls in the lipid solvent tests.

Antisera

Mouse ascitic fluid was prepared by the method of Sartorelli *et al.* (1966) using virus grown in mouse brain.

Specific antisera were prepared in rabbits against certain isolates, including those that were suspected to differ, namely CSIRO 11, CSIRO 58, and CSIRO 82, as well as against D'Aguilar (B8112). The virus used for inoculation was grown in BHK21 tissue cultures using medium 199 with 2% (v/v) rabbit serum as growth medium. The rabbits were given a series of three intramuscular injections with a mixture of virus and Freund's complete adjuvant (Commonwealth Serum Laboratories, Parkville, Vic.) at weekly intervals, and then one intravenous injection with virus alone 4–6 weeks later. They were bled after 7–10 days and the serum was stored at −20°C. All Palyam group isolates were then tested against these four antisera.

Complement Fixation Tests

Complement fixation tests were performed as described by Lennette and Schmidt (1964), using mouse brain antigen prepared by the method of Clarke and Casals (1958). Anticomplementary controls for both antigen and ascitic fluids were included in each test. A non-specific antigen was also titrated in each test in the same manner as the viral antigen.

Neutralization Tests

Neutralization tests, both for identification of all Palyam group isolates and for testing of sera for antibody surveys, were carried out by a micro method in Vero (Commonwealth Serum Laboratories, Parkville, Vic.) cells described by Cybinski *et al.* (1978).

Treatment with Lipid Solvents

Ether and chloroform sensitivity tests were carried out using a modification of the method used by Feldman and Wang (1961). A 1 in 10 dilution of virus in Hanks' balanced salt solution was incubated with 10% (v/v) solvent for 1 h at room temperature. The ether was then allowed to evaporate and the chloroform removed by centrifugation, before titrating the virus in BHK21 tissue culture tubes.

Serological Surveys

The cattle sera used for antibody surveys were from a sentinel herd scheme (St George 1980). The sera tested for antibodies against D'Aguilar (B8112) were from 275 animals from 17 herds bled between one and nine times (average three) at weekly or monthly intervals. In all, 255 animals from 15 herds bled an average of 3·5 times were tested for antibodies to CSIRO 11 virus, 1630 animals from 90 herds bled an average of four times were tested for antibodies against CSIRO 58 virus, and 165 animals from nine herds were bled an average of three times and tested for antibodies to CSIRO 82. All the sera were collected between 1975 and 1980. Sera from other species were also collected from various parts of Australia and these were tested for antibodies to the four viruses.

Results

Isolation of Viruses

All Palyam group viruses isolated between 1974 and 1976, as well as all subsequent isolations of members of the group, are summarized in Table 1. Of a total of 424 virus isolates at this laboratory in 6 years, 165 (39%) belonged to the Palyam group.

Table 1. Classification, by virus neutralization, of Palyam group viruses isolated from different sources at the CSIRO Long Pocket Laboratories

Type strain	Number of isolates			
	Bovine blood	*C. brevitarsis*	Other *Culicoides* spp.	Total
D'Aguilar (B8112[A])	12	1	0	13
CSIRO 11[B]	27	38	0	65
CSIRO 58[C]	73	12	1[D]	86
CSIRO 82[B]	0	0	1[E]	1
Total	112	51	2	165

[A] Doherty *et al.* (1972).
[B] H. A. Standfast and A. L. Dyce (unpublished data).
[C] St George (unpublished data).
[D] Isolated from *C. schultzei.*
[E] Isolated from an insect pool containing *C. schultzei* and *C. peregrinus.*

Of the Palyam group isolates, 112 were from the blood of sentinel animals, and 53 were from *Culicoides* spp. There were instances when the same serotype of virus was isolated two or three times from the same animal. For CSIRO 58 there were five such cases recorded with the interval between isolations ranging from 1 to 8 days, two occasions for CSIRO 11, both after 8 days, and one only for D'Aguilar after 6 days. No Palyam group viruses were isolated from the nine herds south of the *C. brevitarsis* line (Fig. 1).

Different serotypes of Palyam group viruses were sometimes isolated in succession from the same animal, which has previously been noted by St George *et al.* (1979). Of the possible permutations of two serotypes from cattle, four have so far been detected namely CSIRO 11 and CSIRO 58, CSIRO 58 and CSIRO 11, D'Aguilar

and CSIRO 58, and CSIRO 58 and D'Aguilar. No instances have been found where more than two serotypes have been isolated from a single animal.

Serology

The four type strains are not distinguishable by complement fixation (Table 2), but can easily be separated from each other by microneutralization tests (Table 3). Although CSIRO 11 virus was not completely neutralized by antiserum against D'Aguilar virus, more than 50% retardation of cytopathic effect up to a serum dilution of 1 in 16 was noted. In addition, three serotypes were not neutralized by antiserum against Palyam virus (Table 3) although a titre of 6 was obtained with CSIRO 11 virus. However, the titre of this serum against its homologous virus is not known.

Table 2. Complement fixation titres for Australian Palyam group viruses and mouse ascitic fluid prepared against them

Results presented as titre of mouse ascitic fluid/titre of antigen

Antigen	Mouse ascitic fluid against			
	D'Aguilar (B8112)	CSIRO 11	CSIRO 58	CSIRO 82
D'Aguilar (B8112)	*256/16*	256/128	512/⩾128	256/128
CSIRO 11	64/16	*512/⩾128*	512/⩾128	256/⩾128
CSIRO 58	64/16	256/128	*⩾1024/⩾128*	256/128
CSIRO 82	64/8	128/32	512/64	*512/128*

In all cases where a virus was isolated from an animal, specific antibody to this serotype was first detected in the animal between 0 and 21 days after the first isolation date, and after more than one serotype had been isolated, antibodies to each of these serotypes could be demonstrated. On at least three occasions, a particular serotype (D'Aguilar, CSIRO 11 or CSIRO 58 viruses) was isolated from cattle blood despite the presence of homologous antibody to that serotype in the animals.

Table 3. Cross neutralization tests between Australian Palyam group viruses

Virus[A]	Antiserum				
	D'Aguilar (B8112)	CSIRO 11	CSIRO 58	CSIRO 82	Palyam
D'Aguilar (B8112)	512[B]	<2	<2	<2	<2
CSIRO 11	<2	4096	<2	<2	6
CSIRO 58	<2	<2	1024	<2	<2
CSIRO 82	<2	<2	<2	4910	<2

[A] All viruses received four passages in BHK21 cells.

[B] Reciprocal of antiserum dilution that neutralized the virus in 50% of the wells.

Antibody surveys on serially bled cattle gave ample indication that all serotypes except CSIRO 82 may pass through herds in sequence, with individual animals showing evidence of infection with up to three serotypes. The order of infection varied in different seasons, and even in different herds. A virus was not isolated on every occasion where antibody was detected.

Lipid Solvents

The sensitivity of the four Palyam group isolates to ether and chloroform is shown in Table 4. They were found to be more sensitive to chloroform than to ether, but were not completely inactivated by either solvent. A known sensitive virus (MVE)

Table 4. Sensitivity of Palyam group viruses to lipid solvents

Virus	Control titre	Log titre after treatment with	
		Ether	Chloroform
D'Aguilar (B8112)[A]	4·0	2·5	2·0
CSIRO 11[A]	3·5	3·0	2·5
CSIRO 58[A]	4·0	2·5	2·0
CSIRO 82[A]	4·0	3·5	2·5
CSIRO 947 (Reovirus type 2)[B]	3·5	4·0	3·5
MVE[B]	6·5	1·0	0·5

[A] Virus had previously been given four passages in BHK21 tissue culture.
[B] Virus had previously been passaged once in BHK21 tissue culture.

and a known resistant virus (Reovirus type 2) were included as controls. The Reovirus was completely resistant to the treatment with solvents, whereas MVE virus showed a marked reduction in titre to both solvents.

Antibody Distribution—Geographic

Fig. 1 shows the distribution of sentinel herds in Australia and Papua New Guinea that were tested for antibodies to the Palyam group viruses, the approximate southern limits of *C. brevitarsis* as shown by Dyce and Standfast (1979) and the

Table 5. Antibodies against four Palyam group viruses in serum from cattle and other species

	D'Aguilar (B8112)	CSIRO 11	CSIRO 58	CSIRO 82
Cattle	390/811	549/916	3616/6490	2/495[A]
Buffalo	3/16	5/16	14/16	22/26
Sheep	13/72	1/72	42/188	0/14[B]
Deer	1/10	0/13	4/17	0/9[B]
Human	0/29	0/29	0/66	0/29[B]
Horse	0/74	0/83	0/193	0/58
Pig	0/11	0/11	0/24	0/11
Kangaroo	0/100	0/100	0/102	0/4[B]
Possum	0/41	0/41	0/41	0/17[B]
Wallaby	0/5	0/5	0/9	0/5

[A] Two sera from the same animal taken 7 days apart.
[B] No serum available from the area of known distribution.

southern limits of *C. schultzei* and *C. peregrinus* as described by Murray (1975). Antibodies against CSIRO 82 were detected only in serum collected from animals in the far north of the Northern Territory and these show a distribution within that of *C. schultzei* and *C. peregrinus* (Fig. 1). The distribution of neutralizing antibodies

against D'Aguilar, CSIRO 11 and CSIRO 58 viruses in Australian cattle was found to lie within the limits of distribution of *C. brevitarsis*. This is to say that no antibodies to these three viruses were detected in serum from the nine herds south of the *C. brevitarsis* line. All cattle seroconverted to these three viruses in some herds that were bled serially, not only in the north, but in some New South Wales herds as well. However, the incidence of antibodies decreased in sera taken from cattle closer to the borderline of *C. brevitarsis* distribution.

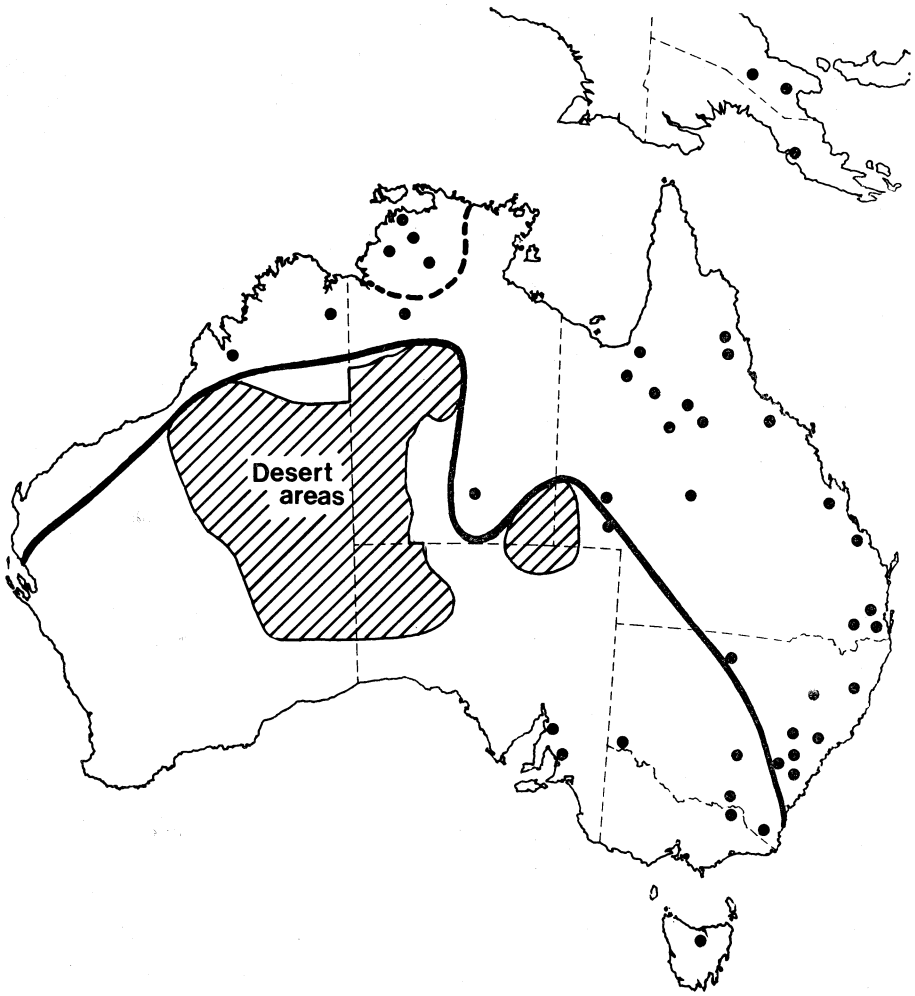

Fig. 1. Distribution of sentinel herds in Australia and Papua New Guinea (●). The areas marked as desert are areas of very low rainfall where there are no cattle or other livestock. —— Approximate southern limits of *Culicoides brevitarsis* (Dyce and Standfast 1979). - - - Approximate southern limits of *C. schultzei* and *C. peregrinus* (Murray 1975).

Antibodies against D'Aguilar, CSIRO 11 and CSIRO 58 viruses, but not CSIRO 82 virus, were detected in cattle serum from Papua New Guinea. No buffalo serum from Papua New Guinea has been tested for antibodies to CSIRO 82 virus.

Antibody Distribution—Species

The occurrences of antibodies against D'Aguilar and the three new Palyam group viruses in humans, various domestic animals and marsupials are shown are Table 5. Only 0·4% of cattle sera tested contained neutralizing antibodies to CSIRO 82 virus, whereas 85% of buffalo sera were found to neutralize this virus.

Discussion

The Palyam group of viruses comprises a large percentage (39%) of all virus isolations made at this laboratory. This may be due to a number of factors including the abundance of this group in the vector–host system under investigation, or the relative efficiency of the isolation techniques for this particular group in comparison with other virus groups.

Although all the Australian members of the Palyam group cross-react markedly in a complement fixation test, a microneutralization test demonstrated quite clearly that the group in fact consists of four distinct serotypes showing little evidence of cross reaction. The reaction of CSIRO 11 virus with antiserum against Palyam virus is difficult to interpret without any knowledge of the antiserum titre to the homologous virus.

Additional evidence for the existence of four distinct Palyam group serotypes in Australia is provided by isolation and antibody studies on cattle infected naturally. The isolation of more than one serotype from the same animal, with corresponding antibody production to these serotypes, is one such piece of evidence. Although all possible sequences of infection have not been demonstrated by virus isolation, the appearance of antibodies to all serotypes except CSIRO 82, in sequence, indicates that infection with any one of these affords either no protection or only short-term protection against infection with the other two serotypes.

Our understanding of the sequence of virus infection and antibody production for the Palyam group in nature is complicated by the apparent occurrence of long periods of viraemia which, for CSIRO 11 and CSIRO 58 viruses, could be demonstrated for up to 8 days. In addition, there was evidence of virus and homologous antibody co-existing in some animals, which confirms the findings of St George and Dimmock (1976). Whether or not the virus may persist long enough in some animals to carry it from one insect season to the next, is a question that still remains to be answered.

Preliminary biochemical studies indicate that the Australian Palyam group viruses are partially resistant to lipid solvents. These results are in agreement with those found for D'Aguilar virus (Doherty *et al.* 1972) and other orbiviruses (Borden *et al.* 1971), and clearly separates this group from other major Arbovirus groups that are solvent sensitive (Borden *et al.* 1971).

Isolates identifiable with the type strains D'Aguilar, CSIRO 11 and CSIRO 58 viruses were all recovered from both bovine blood and *C. brevitarsis* (Table 1). Also, antibody surveys carried out in cattle for these viruses showed a distribution similar to that of *C. brevitarsis* indicating that this insect may be one of the vectors. In addition CSIRO 58 was isolated once from *C. schultzei*, which must be considered as another possible vector for that virus although the distribution of this insect is very limited in comparison with the distribution of the virus as suggested by antibody studies. The likelihood that other vectors exist for these viruses cannot be excluded.

The only insect isolates of Palyam group viruses in Australia and Africa (Lee *et al.* 1974) have been made from *Culicoides* spp., whereas the Indian isolates Palyam, Kasba and Vellore were all recovered from mosquitoes (Dandawate 1974).

The single isolate of CSIRO 82 originated from a pool of *C. schultzei* and *C. peregrinus*, insects that feed preferentially, but not exclusively, on buffalo (Muller *et al.* 1981). Antibodies to this virus have been detected mainly in buffalo serum, although serum from one ox was positive. In contrast, D'Aguilar, CSIRO 11 and CSIRO 58 viruses appear to be associated with a variety of domestic animals, namely cattle, buffalo, deer, and sheep but not humans, horses, or marsupials. The restriction of antibodies to CSIRO 82 virus almost exclusively to buffalo cannot be due solely to the host preference of *C. schultzei* and *C. peregrinus* as these insects do feed on cattle and other domestic animals (Muller *et al.* 1981). However, this feeding preference is probably a contributory factor. The antibody distribution of CSIRO 82 virus is at present only known in the far north of the Northern Territory, the only area sampled where a significant population of water buffalo occurs in Australia.

Many of the animals from which virus was recovered, and also those in whose serum antibody has been detected, have been kept under close surveillance during the period of study, and no evidence of disease associated with these viruses has been detected. In the light of the evidence from Africa, where Nyabira virus was isolated from aborted bovine foetuses, the effects of the Australian isolates on ovine and bovine foetuses should be investigated.

Until recently, the three new Australian viruses have been considered as variants of D'Aguilar virus but they should clearly be known as Palyam group viruses after the first member of the group. The viruses have been tested with antiserum against Palyam virus. They have yet to be compared with the remaining Indian viruses, Kasba and Vellore, and the African viruses Nyabira and Abadina. If they prove to be serologically distinct from these viruses, new names will be proposed.

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