Epidemiol. Infect. (1990), **105**, 447–454
Printed in Great Britain

Isolations of African horse sickness virus from vector insects made during the 1988 epizootic in Spain

P. S. MELLOR[1], J. BONED[2]*, C. HAMBLIN[1] AND S. GRAHAM[1]

[1] *Institute for Animal Health, Pirbright Laboratory, Ash Rd, Pirbright, Woking, Surrey GU24 0NF*

[2] *Junta de Andalucia, Consejeria de Agricultura y Pesca, Direccion General de Agricultura, Ganoderia y Montes, Laboratorio de Sanidad y Produccion Animal, Apartado de Correos 259, 14080 Cordoba, Spain.*

(*Accepted 23 April 1990*)

SUMMARY

This paper describes the first isolations of African horse sickness virus (AHSV) from insects in Spain. Seven isolations of AHSV serotype 4 were made; four from *Culicoides imicola* a known vector of the virus elsewhere, two from mixed pools of *Culicoides* species not including *C. imicola* and one from blood engorged mosquitoes. Three further isolations of AHSV serotype 4 were also made from horses kept adjacent to the insect collecting sites.

This work presents the first definitive identification of the vectors of AHSV in Spain during the 1987, 88 and 89 epizootics. Suggestions are also made concerning the significance of these findings with regard to the epidemiology of African horse sickness in Spain.

INTRODUCTION

African horse sickness virus (AHSV) is a double-stranded RNA virus which causes an infectious arthropod-borne disease of solipeds (horses, mules and donkeys). The disease can be peracute, acute, sub-acute or mild (horse sickness fever). In susceptible populations of horses which can be regarded as an indicator species, the mortality rate may exceed 95% and death may occur within 3 days of infection [1].

There are nine recognized serotypes of AHSV and all are enzootic in tropical and to a lesser extent sub-tropical Africa. However, at intervals the virus makes excursions beyond these enzootic zones to initiate epizootics ranging as far as India and Pakistan in the East [1], Turkey and Cyprus in the North [1, 2] and Morocco and the Cape Verde Isles in the West [2]. The first recorded outbreak of AHS in Spain occurred in early October 1966 during an epizootic of type 9 AHSV which was centred in North Africa [3, 4]. Eight premises were directly affected in Spain but rigorous control measures introduced by the Spanish veterinary authorities ensured that the disease was eradicated by the end of October 1966 [3, 4].

* Present address: Apartado 77, 46980 Paterna, Spain.
Address for correspondence: Dr P. S. Mellor, Institute for Animal Health, Pirbright Laboratory, Ash Road, Pirbright, Woking GU24 0NF.

Subsequent to the 1966 incursion, AHSV was absent from Spain and indeed the whole of Europe for over 20 years. However, in 1982 and 1984 Mellor and his co-workers recorded the widespread presence in Spain of *Culicoides imicola*, the only known field vector of AHSV (B. J. Erasmus, personal communication, 1988) and suggested on epidemiological grounds that the country was vulnerable to AHS incursions up to the latitude of Madrid [5, 6]. This prediction was confirmed in 1987 when a second Spanish incursion of AHSV occurred, this time in the Madrid–Toledo area. The 1987 Spanish AHS outbreak was caused by AHSV type 4, the first occasion on which this virus type had been recorded outside Africa. The probable source of the virus in Spain is considered to be a consignment of five zebra which had been imported into the Madrid area from Namibia just prior to the AHS outbreak [7]. The first death in Spain in 1987 occurred in July but following a vigorous vaccination campaign the outbreak was considered to have been eradicated by 14 December 1987 [8]. However, in October 1988 deaths from AHS were again diagnosed, this time in the Provinces of Cadiz and Malaga in Southern Spain. The causative agent was once again AHSV serotype 4 [9, 10]. Official sources record the last death in this outbreak as being on 8 December 1988 and the officially declared end of the epizootic was 3 April 1989. Unfortunately during August 1989 a further recrudescence of the virus occurred. At the time of writing additional outbreaks are being reported and have caused the deaths of over 1000 equidae in the provinces of Cadiz, Seville, Cordoba, Badajoz and Huelva. The epidemic has also extended to involve areas in the Portuguese Provinces of the Algarve and Baixo Alentejo and northern areas of Morocco.

Previous to the present study and to the work of Mellor and colleagues [5, 6] no information has been published on the identity of the vector or vectors of AHSV in Spain. Therefore, during the course of the 1987 and 1988 AHS epizootics and continuing into 1989, potential vector species of insects were collected in the areas of the epizootics. Virus isolation was attempted from some of these insects. At the same time virus isolation was also attempted from horses kept adjacent to the insect collection sites. This paper, which will be the first of a series concerned with AHSV in Spain, presents the first definitive identification of the Spanish AHSV vectors and records for the first time the isolation of AHSV from vector insects in Spain.

MATERIALS AND METHODS

Insects

Insects were collected for virus isolation attempts during October and November 1988 in the Province of Cadiz. Collections were made in the vicinity of 4 towns – Jimena de la Frontera, San Roque, Vejér de la Frontera and Gaucin, from premises at which AHS had been previously diagnosed.

The insects were collected using Pirbright-type miniature light traps which are a modification of the Monks Wood light trap [11, 12] using a 21 watt automobile bulb as the light source. Traps were operated from dusk to dawn and the insects were blown directly into plastic bottles containing Parke Davis Additive Medium (PDAM) including 0·01 % detergent as a wetting agent plus antibiotics (100 i.u. penicillin and 100 i.u. streptomycin/ml). All insect catches were collected at or

shortly after dawn each day and were maintained in PDAM at 4 °C until preliminary sorting had been carried out – usually within 24 h. The resulting samples of haemotophagous insects were transferred in PDAM into small glass tubes each being labelled with the date and location of collection. These samples were then frozen in liquid nitrogen and transported to the AFRC Institute for Animal Health (IAH), Pirbright, where final sorting was carried out.

Final sorting involved separating insects by date and by location into pools of between 1 and 200 individuals comprising the following categories:

Female mosquito (Aedes) – non-blood-fed
– blood-fed
Female *Culicoides imicola* – non-blood-fed
Female *Culicoides* (other species) – non-blood-fed

The category, female *Culicoides* (other species) consisted of a range of species involving one or more of the following: *C. puncticollis*, *C. pulicaris*, *C. cataneii*, *C. obsoletus*, *C. lailae* and *C. circumscriptus*.

Virus isolation from insects

Virus isolation was attempted by intracerebral inoculation of 3–4 days-old suckling mice with suspensions of triturated insects, after clarification by centrifugation at 2000 rev/min for 5 min. Mice dying within 24 h of inoculation were discarded but the brains of mice dying between 2 and 8 d.p.i. were harvested and ground in buffered bovine albumen at a dilution of 1 brain per 0·5 ml. The brain suspensions were clarified by centrifugation (2000 rev/min for 5 min) and stored at −70 °C until required [13, 14]. Viral agents isolated in this way were adapted to tissue culture by inoculation of mouse brain suspensions onto monolayers of BHK-21 cells in Eagle's growth medium containing 10% foetal calf serum, 1000 units of penicillin and 1000 units of streptomycin per ml [15]. Cell cultures developing typical viral cytopathic effect (CPE) within 2–8 d.p.i. were harvested, clarified by centrifugation at 2000 rev/min for 5 min and the supernatant stored at −70 °C until required.

Virus isolation from horses

Sterile heparinized blood was collected by venous puncture and was maintained at 4 °C until just prior to transport to the IAH, Pirbright when it was frozen in liquid nitrogen. On arrival in the UK the thawed blood was diluted 1 in 10 with sterile phosphate buffered saline (PBS) and then inoculated into the brains of suckling mice (0·02 ml per mouse). Subsequent treatment was identical to that described for the isolation of virus from insects.

Virus identification

The identification of virus isolates was initially carried out by using the group specific AHSV antigen trapping ELISA (C. Hamblin, P. Mertens, P. S. Mellor, J. N. Burroughs and J. Crowther, 1989, unpublished results). Further identification of virus isolates was carried out with the type specific, virus neutralization test using an estimated 1000 $TCID_{50}$ of each test virus against AHSV reference antisera, types 1–9 (adapted from ref 16).

Table 1. *Collections of* Culicoides *made for virus isolation in the Province of Cadiz during October and November 1988*

Date of collection	Site of collection	Species (*Culicoides*)	No. of insects	Pool no.
20.10.88	Esparragal (Jimena de la Frontera)	*imicola*	1	1
21.10.88	Sotogrande (San Roque)	*imicola*	16	2
		pulicaris	5	3
21.10.88	Lomas Est. (Vejer de la Frontera)	*imicola*	10	4
		pulicaris	13	5
		puncticollis	2	
21.10.88	Las Lomas	*imicola*	1	6
21.10.88	Los Pinos	*imicola*	15	7
		puncticollis	3	8
3.11.88	Montenegroli	*imicola*	23	9
		pulicaris	10	10
		cataneii	9	
3.11.88	Montenegroli	*imicola*	100	11
		imicola	123	12
		obsoletus	50	13
		pulicaris	18	
		cataneii	5	
3.11.88	Sotogrande	*imicola*	140	14
		imicola	140	15
		imicola	140	16
		imicola	153	17
5.11.88	Los Pinos	*imicola*	75	18
		pulicaris	4	19
		obsoletus	4	
4.11.88	Montene	*imicola*	10	20
4.11.88	Montenegroli	*imicola*	8	21
		pulicaris	3	22
		lailae	2	
		obsoletus	1	
		cataneii	1	
9.11.88	El Hoyon (Gaucin)	*imicola*	33	23
		pulicaris	193	24
		cataneii	14	25
		obsoletus	12	
		circumscriptus	8	
9.11.88	El Hoyon	*imicola*	30	26
		pulicaris	127	27
		cataneii	20	28
		obsoletus	6	
10.11.88	José Gomez (Gaucin)	*imicola*	3	29
		pulicaris	144	30
		obsoletus	20	31
		cataneii	24	
		circumscriptus	20	

RESULTS

Insects

A total of 1738 non-engorged female *Culicoides*, comprising 7 different species were collected for virus isolation. These insects were divided into 31 pools as shown

Table 2. *Collections of* Mosquitoes *and* Simulium *made for virus isolation in the Province of Cadiz during October and November 1988*

Date of collection	Site of collection	Genus	No. of insects	Pool no.
21.10.88	Sotogrande	*Aedes*	4	32
21.10.88	Los Pinos	*Aedes* (B.F.)	7	33
3.11.88	Montenegroli	*Aedes*	4	34
10.11.88	José Gomez (Gaucin)	*Simulium* (B.F.)	2	35

B.F. Blood fed.

Table 3. *African horse sickness virus isolations from insects in Spain*

Pool no.	Identity of isolate	Passage history	Source of isolate	Comments
2	AHSV type 4	MB_1\BHK5	*C. imicola*	
9	AHSV type 4	MB_1\BHK3	*C. imicola*	
13	AHSV type 4	MB_1\BHK3	*C. obsoletus*, *C. pulicaris*, *C. cataneii*	Mixed pool
17	AHSV type 4	MB_1\BHK4	*C. imicola*	
22	AHSV type 4	MB_1\BHK3	*C. pulicaris*, *C. obsoletus*, *C. lailae*, *C. cataneii*	Mixed pool
23	AHSV type 4	MB_1\BHK3	*C. imicola*	
33	AHSV type 4	MB_1\BHK3	*Aedes* spp.	Blood engorged mosquitoes

on Table 1. At the same time 15 mosquitoes of the genus *Aedes* and 2 *Simulium* spp. were also processed for virus isolation (Table 2). The mosquitoes in pool 33 and the *Simulium* in pool 35 were blood engorged at the time of collection.

All insects were processed in the manner described in the previous section and seven viral agents were isolated, initially in suckling mouse brains. All seven isolates were adapted to growth in BHK-21 cells and were identified as AHSV using the group specific AHSV antigen trapping ELISA. Further identification, involving type specific virus neutralization tests, recorded a $> 2{\cdot}0 \log_{10} TCID_{50}$ reduction in titre of each of the virus isolates in the presence of guinea-pig type 4 hyperimmune anti-AHSV serum. The titres of the seven Spanish virus isolates were unaffected by antisera to the other eight AHSV serotypes. These tests therefore confirmed the identity of each of the seven Spanish isolates as being type 4 AHSV (Table 3).

Horses

Heparinized bloods from nine horses exhibiting clinical signs of AHS were collected during the course of the vector insect survey in Cadiz (Table 4). On intracerebral inoculation into suckling mice two viral agents were initially isolated and were adapted to growth in BHK-21 cells. These agents were identified as being AHSV type 4 by using both the AHSV antigen trapping ELISA and virus neutralization tests. Subsequently the presence of AHSV antigen was detected by the antigen trapping ELISA in the blood of a third horse (Smocky). On re-

Table 4. *African horse sickness virus isolations from Spanish horses*

Location	Date	Horse name	Virus isolation	Passage history
Cadiz – Sotogrande	Nov. 1988	Credi	—	
		Ricarda	AHSV type 4	MB_1\BHK4
		Negrita	—	
		La Almoraima	—	
		Redonda	—	
		Smocky	AHSV type 4*	MB_1\BHK2
		Syrspan	AHSV type 4	MB_1\BHK4
		Gareta	—	
		Cocki	—	

* Isolation only subsequent to detection by the antigen trapping ELISA and on re-inoculation into mice.

inoculation of suckling mice with aliquots of this blood a third isolate of AHSV type 4 was made (Table 4).

DISCUSSION

At the time of the first epizootic of AHSV in Spain (October 1966) it was not possible to speculate on the identity of the vector species of insects involved. However subsequent to that outbreak the widespread presence of populations of *C. imicola*, the only confirmed field vector of AHSV, were reported in Southern Spain and Portugal and it was suggested that this species was the likely vector of AHS in Spain [5, 6].

The present work, recording for the first time in Spain the isolation of AHSV from *C. imicola*, confirms the status of this midge as a vector of AHS. The fact that four separate isolations of AHSV were made from this species of midge and also that at the same time three isolations of the same virus type were made from horses adjacent to the insect collection sites strongly suggests that *C. imicola* is the major vector of AHSV in the area. The presence of this midge therefore obviously constitutes a grave threat to any nearby population of susceptible equines.

Three other isolations of AHSV type 4 were also made from insects during the present study. The isolation from *Aedes* mosquitoes is difficult to interpret in terms of its significance since the insects involved were engorged with fresh blood at the time of capture. The presence of virus may therefore reflect merely a recent blood meal upon a viraemic equine. However, the fact that mosquitoes do acquire AHSV from viraemic hosts in this way means that they are *potentially* capable of transmitting the virus, either biologically as Ozawa and Nakata [17] and Ozawa and colleagues [18, 19] suggested or possibly even mechanically. The likelihood of mosquitoes being involved in AHS transmission, even in a minor role, should not therefore be completely discarded.

Two isolations of AHSV type 4 were also made from mixed pools of *Culicoides* consisting mainly of *C. pulicaris* and *C. obsoletus* (Table 2). This is the first occasion on which AHSV has been isolated from species of *Culicoides* other than *C. imicola*. Previously AHSV has been recovered in the field either from pools consisting only of *C. imicola* (B. J. Erasmus 1988, personal communication) or from mixed pools of *Culicoides* including *C. imicola* [20]. However in the present work the isolation

of AHSV type 4 from mixed pools of *Culicoides* clearly does not involve *C. imicola* at all, since on the two occasions this was achieved (Montenegroli, 3 November 88 and 4 November 88) virus was not isolated from pools of *C. imicola* derived from the same insect catches (Table 1). The significance of this finding could be of considerable importance; while *C. imicola* is basically a tropical and sub-tropical species, the range of both *C. pulicaris* and *C. obsoletus* extends much further north and indeed these two species are probably the commonest midges in Northern Europe and the UK. Intuitively one feels that if either or both of these species of *Culicoides* are involved in AHSV transmission then they are likely to be of less importance than *C. imicola*. However this assessment is based mainly upon the absence of any previous records linking *C. obsoletus* and *C. pulicaris* with AHS. Since AHS only rarely penetrates as far north as Spain it may be that the paucity of evidence linking *C. obsoletus* and *C. pulicaris* with this virus has more to do with a lack of opportunity rather than vector incompetence. Furthermore it is well documented that different populations of a vector species of *Culicoides* can vary widely in their ability to transmit a particular virus [21, 22] therefore some European populations of *C. pulicaris* and *C. obsoletus* may prove to be more efficient AHSV vectors than populations of the same species of midge further south. It is also the case that species of *Culicoides* such as *C. imicola* and *C. variipennis* which are known to be able to transmit AHSV [14, 20, 23, 24] are also competent to transmit the closely related bluetongue viruses [20, 25, 26]. If it should be the case that these two groups of viruses usually share common vectors then it may be of importance to note that both *C. pulicaris* and *C. obsoletus* have been implicated as potential vectors of bluetongue virus [27, 28].

It is clearly of major importance in the light of the recent outbreaks of AHS in Spain that the distribution, prevalence and seasonal incidence of all potential vector species of *Culicoides*, particularly *C. imicola*, should be elucidated as soon as possible. This will enable areas of the country which are at risk to AHS incursion to be identified. Equally important it will also enable those areas which are not at risk to be recognized. Further, it is now known that *C. imicola* is able to survive 'on the wing' throughout the year in certain areas of Spain (P. S. Mellor and J. Boned, unpublished data). Since these areas are likely to provide over-wintering foci for AHSV it is of considerable international importance that their location and boundaries be documented without delay. It will then be possible to implement effective control measures to eliminate all risk of AHS becoming enzootic in Spain.

ACKNOWLEDGEMENTS

The authors are grateful to P. Mertens and J. N. Burroughs for donating the purified infectious sub-viral particles of AHS virus type 9 used for preparing the ELISA reagents.

REFERENCES

1. Rafyi A. Horse sickness. Bull Off Int Epiz 1961; **56**: 216–50.
2. Sellers RF, Pedgley DE, Tucker MR. Possible spread of African horse sickness on the wind. J Hyg 1977; **79**: 279–88.
3. Diaz Montilla R, Panos Marti P. Epizootologia de la peste equina en Espana. Bull Off Int Epiz 1967; **68**: 705–14.

4. Diaz Montilla R, Panos Marti P. La peste équine. Bull Off Int Epiz 1968; **70**: 647–62.
5. Mellor PS, Boorman JPT, Wilkinson PJ, Martinez-Gomez F. Potential vectors of bluetongue and African horse sickness viruses in Spain. Vet Rec 1983; **112**: 229–30.
6. Mellor PS, Jennings DM, Wilkinson PJ, Boorman JPT. *Culicoides imicola*: A bluetongue virus vector in Spain and Portugal. Vet Rec 1985; **116**: 589–90.
7. Lubroth J. African horse sickness and the epizootic in Spain 1987. Equine Practice 1988; **10**: 26–33.
8. Yubero MAD. African horse sickness in Spain: Post-eradication control programme. OIE Epizootiological Information 1988a, Feb 25; No esp.
9. Yubero MAD. African horse sickness in Spain. OIE Disease Information 1988b, 21 Oct; Vol 1: No 2, p 34.
10. Yubero MAD. African horse sickness in Spain. OIE Disease Information 1988c, 4 Nov; Vol 1: No 13, p 37–38.
11. Service MW. A battery-operated light-trap for sampling mosquito populations. Bull WHO 1970; **43**: 635–41.
12. Youdeowei A, Service MW, eds. *Pest and vector management in the tropics*. London: Longman, 1983.
13. Boorman J, Mellor PS. Notes on *Culicoides* (Diptera, Ceratopogonidae) from Sudan in relation to the epidemiology of bluetongue virus disease. Rev Elev Méd Vét Pays Trop 1982; **35**: 173–8.
14. Mellor PS, Boorman J, Jennings M. The multiplication of African horse-sickness virus in two species of *Culicoides* (Diptera, Ceratopogonidae). Arch Virol 1975; **47**: 351–6.
15. Mellor PS, Boorman J. Multiplication of bluetongue virus in *Culicoides nubeculosus* (Meigen) simultaneously infected with the virus and the microfilariae of *Onchocerca cervicalis* (Railliet and Henry). Ann Trop Med Parasitol 1980; **74**: 463–9.
16. Herniman KAJ, Gumm ID, Owen L, Taylor WP, Sellers RF. Distribution of bluetongue viruses and antibodies in some countries of the Eastern Hemisphere. Bull Off Int Epiz 1980; **92**: 581–6.
17. Ozawa Y, Nakata G. Experimental transmission of African horse-sickness by means of mosquitoes. Am J Vet Res 1965; **26**: 744–8.
18. Ozawa Y, Nakata G, Shad-Del F, Navai S. Transmission of African horse-sickness by a species of mosquito *Aedes aegypti*: Linnaeus. Am J Vet Res 1966; **27**: 695–7.
19. Ozawa Y, Shad-Del F, Nakata G, Navai S. Transmission of African horse-sickness by means of mosquito bites and replication of the virus in *Aedes aegypti*. Archives of the Institute, Razi 1970; **22**: 113–22.
20. Du Toit RM. The transmission of bluetongue and horse sickness by *Culicoides*. Onderst J Vet Sci An Indust 1944; **19**: 7–16.
21. Jones RH, Foster NM. Heterogeniety of *Culicoides variipennis* field populations to oral infection with bluetongue virus. Am J Trop Med Hyg 1978; **27**: 178–83.
22. Jennings DM, Mellor PS. The vector potential of British *Culicoides* species for bluetongue virus. Vet Microbiol 1989; **17**: 1–10.
23. Wetzel H, Nevill EM, Erasmus BJ. Studies on the transmission of African horse sickness. Onderst J Vet Res 1970; **37**: 165–8.
24. Boorman J, Mellor PS, Penn M, Jennings M. The growth of African horse sickness virus in embryonated hen eggs and the transmission of virus by *Culicoides variipennis* Coquillett (Diptera, Ceratopogonidae) Arch Virol 1975; **47**: 343–9.
25. Foster NM, Jones RH, Luedke AJ. Transmission of attenuated and virulent bluetongue virus with *Culicoides variipennis* infected orally via sheep. Am J Vet Med 1968; **29**: 275–9.
26. Mellor PS. The replication of bluetongue virus in *Culicoides* vectors. In: Current topics in microbiology and immunology. Bluetongue Viruses 1990; **162**: 143–61.
27. Mellor PS, Pitzolis G. Observations on breeding sites and light-trap collections of *Culicoides* during an outbreak of bluetongue in Greece. Bull Entomol Res 1979; **69**: 229–34.
28. Jennings DM, Mellor PS. Variation in the responses of *Culicoides variipennis* (Diptera, Ceratopogonidae) to oral infection with bluetongue virus. Arch Virol 1987; **95**: 177–82.